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(54) Title: USE OF HEPARIN-BINDING PROTEIN FOR THE MODULATION OR PROPHYLAXIS OF APOPTOSIS OF MAMMALIAN CELLS

(57) Abstract

The present invention relates to the use of a heparin-binding protein for modulating or decreasing apoptosis in mammalian cells of a mammal comprising administering to the mammal an effective amount of a heparin-binding protein or pharmaceutically active fragment thereof.

USE OF HEPARIN-BINDING PROTEIN FOR THE MODULATION OR PROPHYLAXIS OF APOPTOSIS OF MAMMALIAN CELLS

FIELD OF INVENTION

The present invention relates to the use of a heparin-binding protein for modulating or decreasing apoptosis in cells of a mammal, mammalian particularly nerve cells, beta cells from the Islets of Langerhans and endothelial cells comprising administering to the mammal an effective amount of a heparin-binding protein or pharmaceutically active fragment thereof.

BACKGROUND OF THE INVENTION

CELL APOPTOSIS

Cell apoptosis, also known as programmed cell death, is of central importance for the development and homeostasis of metazoan animals. Apoptosis is specifically a process in which, within the limits of a near-to-intact plasma membrane, catabolic enzymes degrade essential macromolecules, leading to a characteristic biochemical and ultrastructural death phenotype of cells (reviewed in Kroemer et al., 1997, Immunol. Today 18:44-51, Zamzami et al., 1997, J. Bioenergetics and Biomembranes, 29:185-193). Specifically, cells receive various death inducing stimuli such as lack of obligatory survival factors, shortage of metabolic supply, ligation of death-signal-transmitting receptors (such as Fas/APO-1/CD95 and tumor necrosis factor receptors, contradictory signal combinations or subnecrotic damage by toxins, heat or irradiation. These stimuli can trigger mitochondrial permeability transition (PT), which involves the formation of pores. These pores appear to consist of several proteins located in the inner and outer mitochondria membranes which cooperate with each other at the contact sites at contact sites where the two membranes come into close apposition. As a result, there is mitochondrial dysfunction, specifically the collapse of mitochondrial transmembranne potential, uncoupling of the respiratory chain, release of soluble intermembrane proteins, outflow of matrix calcium and glutathione, disruption of mitochondrial biogenesis and hyperproduction of superoxide anions. This mitochondrial dysfunction can cause activation and action of apoptogenic proteases with secondary endonuclase activation and consequently oligonu-

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cleosomal DNA fragmentation. It is thought that cytochrome c may play a role in the activation of apoptogenic proteases (Reed, 1997, Cell 91:559-562).

Apoptosis can serve as a prominent force in sculpting the developing organism, as a major mechanism for the precise regulation of cell numbers and as a defense mechanism to remove unwanted and potentially dangerous cells. However, in addition to the beneficial effects of programmed cell death, the inappropriate activation of apoptosis may cause or contribute to a variety of disorders (reviewed in Thompson, 1995, Science 267:1456-1461). These include virus-induced lymphocyte depletion (AIDS); cell death in neurodegenerative disorders char-~~acterized by the gradual loss of specific sets of neurons (e.g., Alzheimer's Disease, Parkin~~-son's disease, ALS, retinitis pigmentosa, spinal muscular atrophy and various forms of cerebellar degeneration), cell death in blood cell disorders resulting from deprivation of growth factors (anemia associated with chronic disease, aplastic anemia, chronic neutropenia and myelodysplastic syndromes) and disorders arising out of an acute loss of blood flow (e.g., myocardial infarctions and stroke).

ANTIAPOPTOSIS AGENTS

It is thought that treatments that can increase the apoptotic thresholds of specific cells may be beneficial in treatment of cell loss associated disorders (reviewed in Thompson, 1995, Science 267:1456-1461). Examples of such treatments may be physiological inhibitors of apoptosis such as growth factors, extracellular matrix, CD40 ligand, neutral amino acids, zinc, estrogen, androgens. Alternatively, pharmacological agents such as calpain inhibitors, cysteine protease inhibitors, tumor promoters such as PMA, phenobarbital, alpha-hexachlorocyclohexane have been thought to act as inhibitors of apoptosis.

DIABETES MELLITUS

Diabetes mellitus is a systemic disease characterized by disorders in the actions of insulin and other regulatory hormones in the metabolism of carbohydrates, fats and proteins and in the structure and function of blood vessels. The primary symptom of diabetes is hyperglycemia, often accompanied by glucosuria the presence in urine of large amounts of glucose and polyuria, the excretion of large volumes of urine. Additional symptoms arise in chronic or

long standing diabetes. These symptoms include degeneration of the walls of blood vessels. Although many different organs are affected by these vascular changes, the nerves eyes and kidneys appear to be the most susceptible. As such, long-standing diabetes mellitus, even when treated with insulin, is a leading cause of blindness.

There are two recognized types of diabetes. Type I diabetes is of juvenile onset, ketosis-prone, develops early in life with much more severe symptoms and has a near-certain prospect of later vascular involvement. Control of this type of diabetes is difficult and requires exogenous insulin administration. Type II diabetes is of adult onset, ketosis-resistant, develops later in life is milder and has a more gradual onset.

One of the most significant advances in the history of medical science came in 1922 when Banting and Best demonstrated the therapeutic effects of insulin in diabetic dogs. However, even today, a clear picture of the basic biochemical defects of the disease is not known and diabetes remains a serious health problem. It is believed that two percent of the United States' population is afflicted with some form of diabetes. The introduction of orally effective hypoglycemic agents was an important development in the treatment of hyperglycemia. Oral hypoglycemic agents are normally used in the treatment of adult onset diabetes.

HEPARIN-BINDING PROTEIN

The covalent structure of two closely related proteins isolated from peripheral neutrophil leukocytes of human and porcine origin have recently been determined (cf. H. Flodgaard et al., 1991, Eur. J. Biochem. 197: 535-547; J. Pohl et al., 1990, FEBS Lett. 272: 200 ff.). Both proteins show a high similarity to neutrophil elastase, but owing to selective mutations of the active serine 195 and histidine 57 (chymotrypsin numbering (B.S. Hartley, "Homologies in Serine Proteinases", Phil. Trans. Roy. Soc. Series 257, 1970, p. 77 ff.)) the proteins lack protease activity. The proteins have been named human heparin-binding protein (hHBP) and porcine heparin-binding protein (pHBP), respectively, owing to their high affinity for heparin; Schafer et al. (W.M. Schafer et al., Infect. Immun. 53, 1986, p. 651 ff.) have named the protein cationic antimicrobial protein (CAP37) due to its antimicrobial activity.

HBP was originally studied because of its antibiotic and LPS binding properties (Gabay et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:5610-5614 and Pereira et al., 1993, Proc. Natl. Acad. Sci. USA 90: 4733-7). However, accumulating evidence now supports the concept that HBP, in addition to its bactericidal role, is involved in the progression of inflammation due to its effect on the recruitment and activation of monocytes (Pereira et al., 1990, J. Clin. Invest. 85:1468-1476 and Rasmussen et al., 1996, FEBS Lett. 390:109-112), recruitment of T cells (Chertov et al., 1996, J. Biol. Chem. 271: 2935-2940), as well as on the induced contraction in endothelial cells and fibroblasts (Ostergaard and Flodgaard, 1992, J. Leuk. Biol. 51: 316 323). Ostergaard and Flodgaard, 1992, op. cit. Also disclose increased survival of monocytes treated with heparin-binding protein but do not speculate on the underlying mechanisms of this increased survival. Furthermore, in animal models of fecal peritonitis, HBP treatment has been shown to rescue mice from an otherwise lethal injury (Mercer-Jones et al., 1996, In Surgical Forum, pp. 105-108 and Wickel et al., 1997, In 4th International Congress on the Immune Consequences of Trauma, Chock and Sepsis, Munich, Germany, pp. 413-416).

From the azurophil granules, a protein with the first 20 N-terminal amino acid residues identical to those of hHBP and CAP37 called azurocidin has also been isolated (J.E. Gabay et al., Proc. Natl. Acad. Sci. USA 86, 1989, p. 5610 ff.; C.G. Wilde et al., J. Biol. Chem. 265, 1990, p. 2038 ff.) and its antimicrobial properties have been reported (D. Campanelli et al., J. Clin. Invest. 85, 1990, p. 904 ff.).

The structure of HBP appears from WO 89/08666 and H. Flodgaard et al., op. cit. HBP has otherwise been termed CAP37 (cf. WO 91/00907, U.S. Patent Nos. 5,458,874 and 5,484,885) and azurocidin (cf. C.G. Wilde et al., J. Biol. Chem. 265, 1990, p. 2038).

There is a need for an effective inhibitor of apoptosis that can treat disorders caused by apoptosis but does not cause disorders associated with the inhibition of apoptosis such as cancer, autoimmune disorders such as systemic lupus erythematosus, immune-mediated glomerulonephritis, and viral infections such as herpesviruses, poxviruses, adenoviruses. It is therefore an object of this invention to have an effective inhibitor of apoptosis for the use in treating disorders associated with increased apoptosis.

SUMMARY OF THE INVENTION

It has surprisingly been found that heparin-binding protein (HBP) can decrease apoptosis of mammalian cells. As shown in the example herein, HBP is internalized into the mitochondria, does affect mitochondrial function and specifically PT pore formation.

The invention is directed to a method of modulating or decreasing apoptosis in mammalian cells of a mammal, particularly beta cells of the Islets of Langerhans, nerve cells and endothelial cells, comprising administering to said mammal in need thereof a mammalian heparin-binding protein which in glycosylated form has (i) a molecular weight of about 28 kD as determined by SDS PAGE under reducing conditions; (ii) is stored in the azurophil granules of polymorphonuclear leukocytes and (iii) is a chemoattractant for monocytes or pharmaceutically active fragment thereof or a composition comprising said heparin-binding protein and a pharmaceutically effective carrier in an amount effective to modulate or decrease apoptosis in said cells. Additionally, the invention is directed to the use of said heparin-binding protein (HBP) or fragment thereof for the manufacture of a medicament for the treatment or prophylaxis of a condition resulting from apoptosis as well as its use in treating or preventing said condition.

The invention is further directed to a composition comprising said heparin-binding protein and a mitochondrial matrix targeting protein. As defined herein, a "mitochondrial matrix targeting protein" is a protein that has an N-terminal extension that functions as a targeting signal to the mitochondria. The targeting signal is a highly degenerate sequence having 20-30 residues capable of folding into a positively charged amphiphilic helix. The invention is further directed to uses of these compositions for the manufacture of a medicament for treating or preventing a disorder resulting from cell apoptosis. Furthermore, the invention is directed to a method for modulating and preventing mammalian cell apoptosis and accordingly a disorder resulting from cell apoptosis comprising administering to a mammal an effective amount of said composition.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the effect of human HBP on MTT values in control and cytokine exposed RIN cells.

Figure 2 shows the effect of human HBP on accumulated NO (nitrite) in control and cytokine exposed RIN cells.

Figure 3 shows the release of HBP in the presence of PMA (Figure 3A) or fMLP (Figure 3B) from PMNs in the presence or absence of HUVECs.

~~Figure 4 shows the characterization of the binding of HBP to proteoglycans. Figure 4A shows~~ HBP binding proteoglycans from ^{35}S-labeled endothelial cells are enriched by ion exchange chromatography and affinity purified on HBP-agarose. Fractions, eluted with a stepwise ionic strength gradient (250 - 1000 mM NaCl), are separated on SDS-PAGE and visualized by Phospho-Imaging. Uncoupled agarose is used as a control column. Molecular markers are given on the left. Figure 4B shows ^{35}S-labeled HBP-affinity purified proteoglycans, eluted with 300, 400 and 500 mM NaCl, were treated (+) with CABC or HNO2. Cleavage products were separated on agarose gels and visualized by Phospho-Imaging. As a control, untreated material (-) is analyzed under the same conditions. Figure 4C shows unlabelled endothelial cell proteoglycans eluted from the HBP-column with 300 mM NaCl or endothelial cell lysates, were digested with CABC and heparitinase (+) or left undigested (-). The cleavage products were separated by SDS-PAGE and transferred to Zeta-Probe membranes. Membranes were incubated with mAb 3G10 directed against desaturated glucuronate of heparitinase digested heparan sulfate proteoglycan core proteins. The positions of the different proteoglycans are indicated (*). Molecular markers are given on the left.

Figure 5 shows a characterization of the internalization process.
Endothelial cells are pretreated with diverse substances able to inhibit receptor-ligand interactions, protein synthesis or actin polymerization before addition of HBP. Cells are preincubated with heparin (100 mg/ml) for 30 min. Alternatively, cells are pretreated with NH_4Cl (50 mM), cycloheximide (1 nM) or cytocholasin D (1 mM) for 60 min prior to addition of HBP (50 mg/ml). Bound and/or internalized HBP is quantified in intact or permeabilized cells by FACS analysis. As a positive and negative control respectively, endothelial cells are incu-

bated with HBP or buffer (control) alone followed by incubation with primary and secondary antibodies as described. Data represent the MFI ± SD (n=3). Figure 5B shows CHO wildtype (CHO-K1) and heparan sulfate proteoglycan deficient pgsD 677 cells are treated with HBP for indicated time periods. Cells are permeabilized and analyzed as described above.

Figure 6 shows the subcellular fractionation of HBP-treated HUVECs. Equal amounts of protein from the various cell fractions are subjected to Western blotting using anti-HBP (upper panel) and ani-p33 (lower panel).

Figure 7 shows binding of HBP to rp33. In Figure 7A, microtiter plates are coated with H kininogen, HBP or the control protein KLH (1 mg/ml) followed by the incubation of serial dilutions (starting concentration 2 mg/ml, two fold dilution) of rp33 (fusion protein) or the fusion partner MBP. Bound protein is detected by a rabbit antiserum raised against the fusion partner MBP (1:2500 v/v) and a peroxidase-conjugated secondary antibody against rabbit IgG (1:3000 v/v). The absorption at 405 nm is presented in arbitrary units. Overlay plots of the binding of rp33 to immobilized HBP in the absence (Figure 7B) or presence (Figure 7C) of Zn^{2+} using plasmon resonance spectroscopy. Increasing concentrations of rp33 (6.25, 12.5, 25, 50 or 100 mg/ml) or MBP (100 mg/ml) are applied for 3 min during the association phase followed by injection of buffer alone to monitor the dissociation of formed complexes.

Figure 8 shows colocalization of HBP with p33 and HBP with mitochondria. In Figures 8A and B, endothelial cells are incubated with FITC-labeled HBP (green) for 6 hours, fixed in 4% formaldehyde and incubated with an antibody against rp33 followed by a Texas Red conjugated secondary antibody against rabbit IgG (red). The panel DOUBLE shows an overlay of the FITC and the Texas Red staining. In the panel SUBTRACT, the FITC signal is subtracted from the Texas Red signal. Magnification = 310X. In Figure 8C, endothelial cells are incubated with unconjugated HBP for 30 minutes, fixed in 4% formaldehyde and incubated firstly with a biotinylated rabbit-anti-HBP antibody followed by FITC-streptavidin, and secondly mAb 1273 against human mitochondria followed by a Texas Red conjugated secondary antibody against mouse IgG. Colocalization is shown in the panel DOUBLE and subtraction in panel SUBTRACT. Magnification = 310X.

Figure 9 shows the effect of human heparin-binding protein on apoptosis induced by removal of FCS.

Figure 10 shows the effect of HBP on hydrogen peroxide treated cells.

DETAILED DESCRIPTION OF THE INVENTION

The HBP may suitably be of mammalian, in particular human or porcine, origin. In particular, the HBP is a mature human HBP which has at least about an 80% identity with the amino ~~acid sequence set forth in SEQ ID NO:1, more preferably at least about 90%, even more pref-~~ erably at least about 95%, and most preferably at least about 97% (hereinafter "homologous polypeptides"), which qualitative retain the activity of said heparin-binding protein, or a fragment thereof which inhibits the entry of a pathogen into a mononuclear (e.g., monocyte or macrophage) cells of a patient. Alternatively, the HBP is a mature porcine HBP which has at least about an 80% identity with the amino acid sequence set forth in SEQ ID NO:2, more preferably at least about 90%, even more preferably at least about 95%, and most preferably at least about 97%, which qualitative retain the activity of said heparin-binding protein, or a fragment thereof which inhibits decreases or modulates apoptosis of mammalian cells in a mammal. In a preferred embodiment, the mammal is a human patient.

In a preferred embodiment, the homologous polypeptides have an amino acid sequence which differs by five amino acids, preferably by four amino acids, more preferably by three amino acids, even more preferably by two amino acids, and most preferably by one amino acid from the amino acid sequence set forth in SEQ ID NOS:1 or 2. The degree of identity between two or more amino acid sequences may be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Needleman and Wunsch, 1970, Journal of Molecular Biology 48:443-453). For purposes of determining the degree of identity between two amino acid sequences for the present invention, GAP is used with the following settings: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

The amino acid sequences of the homologous polypeptides differ from the amino acid sequence set forth in SEQ ID NOS: 1 or 2 by an insertion or deletion of one or more amino acid

residues and/or the substitution of one or more amino acid residues by different amino acid residues. Preferably, amino acid changes are of a minor nature, that is, conservative amino acid substitutions that do not significantly affect the folding and/or activity of the protein; small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to about 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the group of basic amino acids (such as arginine, lysine and histidine), acidic amino acids (such as glutamic acid and aspartic acid), polar amino acids (such as glutamine and asparagine), hydrophobic amino acids (such as leucine, isoleucine and valine), aromatic amino acids (such as phenylalanine, tryptophan and tyrosine) and small amino acids (such as glycine, alanine, serine, threonine and methionine). Amino acid substitutions which do not generally alter the specific activity are known in the art and are described, e.g., by H. Neurath and R.L. Hill, 1979, in, The Proteins, Academic Press, New York. The most commonly occurring exchanges are: Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly as well as these in reverse.

The heparin binding protein may be encoded by a nucleic acid sequence having at least about an 80% identity with the nucleic acid sequence set forth in SEQ ID NO:3 (which encodes mature human HBP depicted in SEQ ID NO:1), SEQ ID NO:5 (which encodes a human HBP which includes the pro sequence and sequence of the mature protein, depicted in SEQ ID NO:6), SEQ ID NO:7 (which encodes human HBP which includes the signal sequence, the pro sequence and sequence of the mature protein, depicted in SEQ ID NO:8) or SEQ ID NO:4 (which encodes porcine HBP depicted in SEQ ID NO:2), SEQ ID NO:9 (which encodes a porcine HBP which includes the pro sequence and sequence of the mature protein depicted in SEQ ID NO:10), SEQ ID NO:11 (which encodes porcine HBP which includes the signal sequence, the pro sequence and sequence of the mature protein depicted in SEQ ID NO:12), more preferably at least about 90%, even more preferably at least about 95%, and most preferably at least about 97%, as determined by agarose gel electrophoresis. The nucleic acid se-

quence may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

IVGGRKARPRQFPFLASIQNQGRHFCGGALIHARFVMTAASCFQSQNPGVSTVVLGA
YDLRRRERQSRQTFSISSMSENGYDPQQNLNDLMLLQLDREANLTSSVTILPLPLQNA
TVEAGTRCQVAGWGSQRSGGRLSRFPRFVNVTVTPEDQCRPNNVCTGVLTRRGGIC
NGDGGTPLVCEGLAHGVASFSLGPCGRGPDFFTRVALFRDWIDGVLNNPGPGPA*
(SEQ IDNO:1)

IVGG RRAQPQEFPF LASIQKQGRP FCAGALVHPR FVLTAASCFR GKNSGSASVV
LGAYDLRQQE QSRQTFSIRS ISQNGYDPRQ NLNDVLLLQL DREARLTPSV
ALVPLPPQNA TVEAGTNCQVEAGWGTQRLRR LFSRFPRVLN VTVTSNPCLP
RDMCIGVFSR RGRISQGDRG TPLVCNGLAQ GVASFLRRRF RRSSGFFTRV
ALFRNWIDSV LNNPPA* (SEQ ID NO:2)

ATCGTTGGCGGC CGGAAGGCGA GGCCCCGCCA GTTCCCGTTC
CTGGCCTCCA TTCAGAATCA AGGCAGGCAC TTCTGCGGGG GTGCCCTGAT
CCATGCCCGCTTCGTGATGA CCGCGGCCAG CTGCTTCCAA AGCCAGAACC
CCGGGGTTAG CACCGTGGTG CTGGGTGCCT ATGACCTGAG GCGGCGGGAG
AGGCAGTCCC GCCAGACGTT TTCCATCAGCAGCATGAGCG AGAATGGCTA
CGACCCCCAG CAGAACCTGA ACGACCTGAT GCTGCTTCAG CTGGACCGTG
AGGCCAACCT CACCAGCAGC GTGACGATAC TGCCACTGCC TCTGCA-
GAACGCCACGGTGG AAGCCGGCAC CAGATGCCAG GTGGCCGGCT GGGGGAG-
CCA GCGCAGTGGG GGGCGTCTCT CCCGTTTTCC CAGGTTCGTC AACGTGACTG
TGACCCCCGA GGACCAGTGTCGCCCCAACA ACGTGTGCAC CGGTGTGCTC
ACCCGCCGCG GTGGCATCTG CAATGGGGAC GGGGGCACCC CCCTCGTCTG
CGAGGGCCTG GCCCACGGCG TGGCCTCCTT TTCCCTGGGGCCCTGTGGCC
GAGGCCCTGA CTTCTTCACC CGAGTGGCGC TCTTCCGAGA CTGGATCGAT
GGCGTTTTAA ACAATCCGGG ACCGGGGCCA GCCTAG * (SEQ ID NO:3)

AT TGTGGGCGGC AGGAGGGCCC AGCCGCAGGA GTTCCCGTTT CTGGCCTCCA
TTCAGAAACA AGGGAGGCCC TTTtGCGCCG GAGCCCTGGT CCATCCCCGC

TTCGTCCTGA CAGCGGCCAG CTGCTTCCGT GGCAAGAACA GCGGAAGTGC
CTCTGTGGTG CTGGGGGCCT ATGACCTGAG GCAGCAGGAG CAGTCCCGGC
AGACATTCTC CATCAGGAGC ATCAGCCAGA ACGGCTATGA YCCCCGGCAG
AATCTGAACG ATGTGCTGCT GCTGCAGCTG GACCGTGAGG CCAGACTCAC
CCCCAGTGTG GCCCTGGTAC CGCTGCCCCC GCAGAATGCC ACAGTGGAAG
CTGGCACCAA CTGCCAAGTTGCGGGCTGGG GGACCCAGCG GCTTAGGAGG
CTTTTCTCCC GCTTCCCAAG GGTGCTCAAT GTCACCGTGA CCTCAAACCC
GTGTCTCCCC AGAGACATGT GCATTGGTGT CTTCAGCCGC CGGGGCCGCA
TCAGCCAGGG AGACAGAGGC ACCCCCCTCG TCTGCAACGG CCTGGCGCAG
~~GGCGTGGCCT CCTTCCTCCG GAGGCGTTTC CGCAGGAGCT CCGGCTTCTT~~
CACCCGCGTG GCGCTCTTCA GAAATTGGAT TGATTCAGTT CTCAACAACC
CGCCGGCCTGA* (SEQ ID NO:4)

GGCTCCAGCCCCC TTTTGGAC ATCGTTGGCGGC CGGAAGGCGA GGCCCCGCCA
GTTCCCGTTC CTGGCCTCCA TTCAGAATCA AGGCAGGCAC TTCTGCGGGG
GTGCCCTGAT CCATGCCCGCTTCGTGATGA CCGCGGCCAG CTGCTTCCAA
AGCCAGAACC CCGGGGTTAG CACCGTGGTG CTGGGTGCCT ATGACCTGAG
GCGGCGGGAG AGGCAGTCCC GCCAGACGTT TTCCATCAGCAGCATGAGCG
AGAATGGCTA CGACCCCCAG CAGAACCTGA ACGACCTGAT GCTGCTTCAG
CTGGACCGTG AGGCCAACCT CACCAGCAGC GTGACGATAC TGCCACTGCC
TCTGCAGAACGCCACGGTGG AAGCCGGCAC CAGATGCCAG GTGGCCGGCT
GGGGGAGCCA GCGCAGTGGG GGGCGTCTCT CCCGTTTTCC CAGGTTCGTC
AACGTGACTG TGACCCCCGA GGACCAGTGTCGCCCCAACA ACGTGTGCAC
CGGTGTGCTC ACCCGCCGCG GTGGCATCTG CAATGGGGAC
GGGGGCACCC CCCTCGTCTG CGAGGGCCTG GCCCACGGCG TGGCCTCCTT
TTCCCTGGGGCCCTGTGGCC GAGGCCCTGA CTTCTTCACC CGAGTGGCGC
TCTTCCGAGA CTGGATCGAT GGCGTTTTAA ACAATCCGGG ACCGGGGCCA
GCCTAG * (SEQ ID NO:5)

GSSPLLDIVGGRKARPRQFPFLASIQNQGRHFCGGALIHARFVMTAASCFQSQNPGVS
TVVLGAYDLRRRERQSRQTFSISSMSENGYDPQQNLNDLMLLQLDREANLTSSVTILP
LPLQNATVEAGTRCQVAGWGSQRSGGRLSRFPRFVNVTVTPEDQCRPNNVCTGVLT

RRGGICNGDGGTPLVCEGLAHGVASFSLGPCGRGPDFFTRVALFRDWIDGVLNNPGP GPA*(SEQ ID NO:6)

ATGACCCGGC TGACAGTCCT GGCCCTGCTG GCTGGTCTGC TGGCGTCCTC GAGGGCC GGCTCCAGCCCCC TTTTGGAC ATCGTTGGCGGC CGGAAGGCGA GGCCCCGCCA GTTCCCGTTC CTGGCCTCCA TTCAGAATCA AGGCAGGCAC TTCTGCGGGG GTGCCCTGAT CCATGCCCGCTTCGTGATGA CCGCGGCCAG CTGCTTCCAA AGCCAGAACC CCGGGGTTAG CACCGTGGTG CTGGGTGCCT AT-GACCTGAG GCGGCGGGAG AGGCAGTCCC GCCAGACGTT TTCCATCAGCAG-CATGAGCG AGAATGGCTA CGACCCCCAG CAGAACCTGA ACGACCTGAT GCTGCTTCAG CTGGACCGTG AGGCCAACCT CACCAGCAGC GTGACGATAC TGCCACTGCC TCTGCAGAACGCCACGGTGG AAGCCGGCAC CAGATGCCAG GTGGCCGGCT GGGGGAGCCA GCGCAGTGGG GGGCGTCTCT CCCGTTTTCC CAGGTTCGTC AACGTGACTG TGACCCCCGA GGACCAGTGTCGCCCCAACA ACGTGTGCAC CGGTGTGCTC ACCCGCCGCG GTGGCATCTG CAATGGGGAC GGGGGCACCC CCCTCGTCTG CGAGGGCCTG GCCCACGGCG TGGCCTCCTT TTCCCTGGGGCCCTGTGGCC GAGGCCCTGA CTTCTTCACC CGAGTGGCGC TCTTCCGAGA CTGGATCGAT
GGCGTTTTAA ACAATCCGGG ACCGGGGCCA GCCTAG * (SEQ ID NO:7)

IVGGRKARPRQFPFLASIQNQGRHFCGGALIHARFVMTAASCFQSQNPGVSTVVLGA YDLRRRERQSRQTFSISSMSENGYDPQQNLNDLMLLQLDREANLTSSVTILPLPLQNA TVEAGTRCQVAGWGSQRSGGRLSRFPRFVNVTVTPEDQCRPNNVCTGVLTRRGGIC NGDGGTPLVCEGLAHGVASFSLGPCGRGPDFFTRVALFRDWIDGVLNNPGPGPA* (SEQ ID NO:8)

ATGCCAGCAC TCAGATTCCT GGCCCTGCTG GCCAGCCTGC TGGCAACCTC CAGGGTT AT TGTGGGCGGC AGGAGGGCCC AGCCGCAGGA GTTCCCGTTT CTGGCCTCCA TTCAGAAACA AGGGAGGCCC TTTTGCGCCG GAGCCCTGGT CCATCCCCGC TTCGTCCTGA CAGCGGCCAG CTGCTTCCGT GGCAAGAACA GCGGAAGTGC CTCTGTGGTG CTGGGGGCCT ATGACCTGAG GCAGCAGGAG CAGTCCCGGC AGACATTCTC CATCAGGAGC ATCAGCCAGA ACGGCTATGA

YCCCCGGCAG AATCTGAACG ATGTGCTGCT GCTGCAGCTG GACCGTGAGG CCAGACTCAC CCCCAGTGTG GCCCTGGTAC CGCTGCCCCC GCAGAATGCC ACAGTGGAAG CTGGCACCAA CTGCCAAGTTGCGGGCTGGG GGACCCAGCG GCTTAGGAGG CTTTTCTCCC GCTTCCCAAG GGTGCTCAAT GTCACCGTGA CCTCAAACCC GTGTCTCCCC AGAGACATGT GCATTGGTGT CTTCAGCCGC CGGGGCCGCA TCAGCCAGGG AGACAGAGGC ACCCCCCTCG TCTGCAACGG CCTGGCGCAG GGCGTGGCCT CCTTCCTCCG GAGGCGTTTC CGCAGGAGCT CCGGCTTCTT CACCCGCGTG GCGCTCTTCA GAAATTGGAT TGATTCAGTT CTCAACAACC CGCCGGCCTGA* (SEQ ID NO:9)

MPALRFLALL ASLLATSRV IVGG RRAQPQEFPF LASIQKQGRP FCAGALVHPR FVLTAASCFR GKNSGSASVV LGAYDLRQQE QSRQTFSIRS ISQNGYDPRQ NLNDVLLLQL DREARLTPSV ALVPLPPQNA TVEAGTNCQV AGWGTQRLRR LFSRFPRVLN VTVTSNPCLP RDMCIGVFSR RGRISQGDRG TPLVCNGLAQ GVASFLRRRF RRSSGFFTRV ALFRNWIDSV LNNPPA* (SEQ ID NO:10)

ATG CCAGCAC TCAGATTCCT GGCCCTGCTG GCCAGCCTGC TGGCAACCTC CAGG GTT GGC TTG GCC ACC CTG GCA GAC ATT GTGGGCGGC AGGAGGGCCC AGCCGCAGGA GTTCCCGTTT CTGGCCTCCA TTCAGAAACA AGGGAGGCCC TTTtGCGCCG GAGCCCTGGT CCATCCCCGC TTCGTCCTGA CAGCGGCCAG CTGCTTCCGT GGCAAGAACA GCGGAAGTGC CTCTGTGGTG CTGGGGGCCT AT-GACCTGAG GCAGCAGGAG CAGTCCCGGC AGACATTCTC CATCAGGAGC AT-CAGCCAGA ACGGCTATGA CCCCCGGCAG AATCTGAACG ATGTGCTGCT GCTGCAGCTG GACCGTGAGG CCAGACTCAC CCCCAGTGTG GCCCTGGTAC CGCTGCCCCC GCAGAATGCC ACAGTGGAAG CTGGCACCAA CTGCCAAGTTGCGGGCTGGG GGACCCAGCG GCTTAGGAGG CTTTTCTCCC GCTTCCCAAG GGTGCTCAAT GTCACCGTGA CCTCAAACCC GTGTCTCCCC AGAGACATGT GCATTGGTGT CTTCAGCCGC CGGGGCCGCA TCAGCCAGGG AGACAGAGGC ACCCCCCTCG TCTGCAACGG CCTGGCGCAG GGCGTGGCCT CCTTCCTCCG GAGGCGTTTC CGCAGGAGCT CCGGCTTCTT CACCCGCGTG

GCGCTCTTCA GAAATTGGAT TGATTCAGTT CTCAACAACC CGCCGGCCTGA*(SEQ ID NO:11)

MPALRFLALL ASLLATSRV GLATLAD IVGG RRAQPQEFPF LASIQKQGRP FCAGALVHPR FVLTAASCFR GKNSGSASVV LGAYDLRQQE QSRQTFSIRS ISQNGYDPRQ NLNDVLLLQL DREARLTPSV ALVPLPPQNA TVEAGTNCQV AGWGTQRLRR LFSRFPRVLN VTVTSNPCLP RDMCIGVFSR RGRISQGDRG TPLVCNGLAQ GVASFLRRRF RRSSGFFTRV ALFRNWIDSV LNNPPA* (SEQ ID NO:12)

The degree of identity between two nucleic acid sequences may be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Needleman and Wunsch, 1970, Journal of Molecular Biology 48:443-453). For purposes of determining the degree of identity between two nucleic acid sequences for the present invention, GAP is used with the following settings: GAP creation penalty of 5.0 and GAP extension penalty of 0.3.

Modification of the nucleic acid sequence encoding the HBP may be necessary for the synthesis of polypeptide sequences substantially similar to the HBP. The term "substantially similar" to the HBP refers to non-naturally occurring forms of the HBP. These polypeptide sequences may differ in some engineered way from the HBP isolated from its native source. For example, it may be of interest to synthesize variants of the HBP where the variants differ in specific activity, thermostability, pH optimum, or the like using, e.g., site-directed mutagenesis. The analogous sequence may be constructed on the basis of the nucleic acid sequence presented as the HBP encoding part of SEQ ID NOS:1, 2, 6, 8, 10, or 12, e.g., a subsequence thereof, and/or by introduction of nucleotide substitutions which do not give rise to another amino acid sequence of the HBP encoded by the nucleic acid sequence, but which corresponds to the codon usage of the host organism intended for production of the enzyme, or by introduction of nucleotide substitutions which may give rise to a different amino acid sequence. For a general description of nucleotide substitution, see, e.g., Ford ET al., 1991, in Protein Expression and Purification 2:95-107.

It will be apparent to those skilled in the art that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active polypeptide sequence. Amino acid residues essential to the activity of the polypeptide encoded by the isolated nucleic acid sequence of the invention, and therefore preferably not subject to substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (see, e.g., Cunningham and Wells, 1989, Science 244:1081 1085). In the latter technique mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for HBP activity to identify amino acid residues that are critical to the activity of the molecule.

The heparin-binding protein may also be encoded by a nucleic acid sequence that hybridizes to a nucleic acid sequence set forth in SEQ ID NOS: 3, 4, 6, 8, 10, and 12 at low to high stringency conditions. Low to high stringency conditions are defined as prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 ug/ml sheared and denatured salmon sperm DNA and either 25, 35 or 50% formamide for low, medium and high stringencies, respectively. The carrier material is washed three times each for 30 minutes using 2X SSC, 0.2% SDS preferably at least at 50°C (very low stringency), more preferably at least at 55°C (low stringency), more preferably at least at 60°C (medium stringency), more preferably at least at 65°C (medium-high stringency), even more preferably at least at 70°C (high stringency) and most preferably at least at 75°C (very high stringency).

PREPARATION OF HBP

A nucleic acid sequence encoding HBP may be prepared synthetically by established standard methods, e.g., the phosphoamidite method described by S.L. Beaucage and M.H. Caruthers, Tetrahedron Letters 22, 1981, pp. 1859-1869, or the method described by Matthes et al., EMBO Journal 3, 1984, pp. 801-805. According to the phosphoamidite method, oligonucleotides are synthesized, e.g., in an automatic DNA synthesizer, purified, annealed, ligated and cloned in suitable vectors.

The techniques used to isolate or clone a nucleic acid sequence encoding the heparin binding protein used in the method of the present invention are known in the art and include isolation

from genomic DNA, preparation from cDNA, or a combination thereof. The cloning of the nucleic acid sequences of the present invention from such genomic DNA can be effected, e.g., by using the well known polymerase chain reaction (PCR) or antibody screening of expression libraries to detect cloned DNA fragments with shared structural features. See, e.g., Innis et al., 1990, A Guide to Methods and Application, Academic Press, New York. Other nucleic acid amplification procedures such as ligase chain reaction (LCR), ligated activated transcription (LAT) and nucleic acid sequence-based amplification (NASBA) may be used.

The nucleic acid sequence is then inserted into a recombinant expression vector which may be ~~any vector which may conveniently be subjected to recombinant DNA procedures. The choice~~ of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e., a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the nucleic acid sequence encoding HBP should be operably connected to a suitable promoter sequence. The promoter may be any nucleic acid sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the nucleic acid sequence encoding HBP in mammalian cells are the SV 40 promoter (Subramani et al., Mol. Cell Biol. 1, 1981, pp. 854-864), the MT-1 (metallothionein gene) promoter (Palmiter et al., Science 222, 1983, pp. 809-814) or the adenovirus 2 major late promoter, a Rous sarcoma virus (RSV) promoter, cytomegalovirus (CMV) promoter (Boshart et al., 1981, Cell 41:521-530) and a bovine papilloma virus promoter (BPV). A suitable promoter for use in insect cells is the polyhedrin promoter (Vasuvedan et al., FEBS Lett. 311, 1992, pp. 7-11).

Examples of suitable promoters for directing the transcription of the nucleic acid sequence encoding HBP, especially in a bacterial host cell, are the promoters obtained from the E. coli lac operon, the Streptomyces coelicolor agarase gene (dagA), the Bacillus subtilis levansu-

crase gene (sacB), the Bacillus licheniformis alpha-amylase gene (amyL), the Bacillus stearothermophilus maltogenic amylase gene (amyM), the Bacillus amyloliquefaciens alpha amylase gene (amyQ), the Bacillus licheniformis penicillinase gene (penP), the Bacillus subtilis xylA and xylB genes, and the prokaryotic beta-lactamase gene (Villa-Kamaroff et al., 1978, Proceedings of the National Academy of Sciences USA 75:3727-3731), as well as the tac promoter (DeBoer et al., 1983, Proceedings of the National Academy of Sciences USA 80:21 25). Further promoters are described in "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; and in Sambrook et al., 1989, supra.

Examples of suitable promoters for directing the transcription of the nucleic acid sequence encoding HBP in a filamentous fungal host cell are promoters obtained from the genes encoding *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (glaA), *Rhizomucor miehei* lipase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Aspergillus nidulans* acetamidase, *Fusarium oxysporum* trypsin-like protease (as described in U.S. Patent No. 4,288,627, which is incorporated herein by reference), and hybrids thereof. Particularly preferred promoters for use in filamentous fungal host cells are the TAKA amylase, NA2-tpi (a hybrid of the promoters from the genes encoding Aspergillus niger neutral a amylase and *Aspergillus oryzae* triose phosphate isomerase), and glaA promoters.

In a yeast host, useful promoters are obtained from the *Saccharomyces cerevisiae* enolase (ENO-1) gene, the *Saccharomyces cerevisiae* galactokinase gene (GAL1), the *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase genes (ADH2/GAP), and the *Saccharomyces cerevisiae* 3-phosphoglycerate kinase gene. Other useful promoters for yeast host cells are described by Romanos et al., 1992, Yeast 8:423-488.

The nucleic acid sequences encoding SEQ ID NOS: 1 and 2, e.g., SEQ ID NOS:3 and 9 may be operably linked to a nucleic acid encoding a heterologous pro sequence. The nucleic acid encoding SEQ ID NOS:6, 8, 10, and 12, e.g., SEQ ID NOS:5, 7, 9, and 11 and may be operably linked to a nucleic acid sequence encoding a heterologous signal sequence and/or pro sequence.

The nucleic acid sequence encoding HBP may also be operably connected to a suitable terminator, such as the human growth hormone terminator (Palmiter et al., op. cit.) Preferred terminators for filamentous fungal host cells are obtained from the genes encoding *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* alpha-glucosidase, and *Fusarium oxysporum* trypsin-like protease.

Preferred terminators for yeast host cells are obtained from the genes encoding *Saccharomyces cerevisiae* enolase, *Saccharomyces cerevisiae* cytochrome C (CYC1), or *Saccharomyces cerevisiae* glyceraldehyde-3-phosphate dehydrogenase. Other useful terminators for yeast host cells are described by Romanos et al., 1992, supra.

The vector may further comprise elements such as polyadenylation signals (e.g. from SV 40 or the adenovirus 5 Elb region), transcriptional enhancer sequences (e.g. the SV 40 enhancer) and translational enhancer sequences (e.g. the ones encoding adenovirus VA RNAs). Furthermore, preferred polyadenylation sequences for filamentous fungal host cells are obtained from the genes encoding Aspergillus oryzae TAKA amylase, Aspergillus niger glucoamylase, Aspergillus nidulans anthranilate synthase, and Aspergillus niger alpha-glucosidase. Useful polyadenylation sequences for yeast host cells are described by Guo and Sherman, 1995, Molecular Cellular Biology 15:5983-5990.

The recombinant expression vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such a sequence (when the host cell is a mammalian cell) is the SV 40 or polyoma origin of replication. Examples of bacterial origins of replication are the origins of replication of plasmids pBR322, pUC19, pACYC177, pACYC184, pUB110, pE194, pTA1060, and pAMß1. Examples of origin of replications for use in a yeast host cell are the 2 micron origin of replication, the combination of CEN6 and ARS4, and the combination of CEN3 and ARS1. The origin of replication may be one having a mutation to make its function temperature-sensitive in the host cell (see, e.g., Ehrlich, 1978, Proceedings of the National Academy of Sciences USA 75:1433).

The vector may also comprise a selectable marker, e.g., a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or one which confers resistance to a drug, e.g., neomycin, geneticin, ampicillin, or hygromycin. Suitable markers for yeast host cells are ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3. A selectable marker for use in a filamentous fungal host cell may be selected from the group including, but not limited to, amdS (acetamidase), argB (ornithine carbamoyltransferase), bar (phosphinothricin acetyltransferase), hygB (hygromycin phosphotransferase), niaD (nitrate reductase), pyrG (orotidine-5'-phosphate decarboxylase), sC (sulfate adenyltransferase), trpC (anthranilate synthase), and glufosinate resistance markers, as well as ~~equivalents from other species. Preferred for use in an Aspergillus cell are the amdS and~~ pyrG markers of *Aspergillus nidulans* or *Aspergillus oryzae* and the bar marker of Streptomyces hygroscopicus. Furthermore, selection may be accomplished by co transformation, e.g., as described in WO 91/17243, where the selectable marker is on a separate vector.

The procedures used to ligate the nucleic acid sequences coding for HBP, the promoter and the terminator, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., op.cit.).

The host cell into which the expression vector is introduced may be any cell which is capable of producing HBP and is preferably a eukaryotic cell, such as invertebrate (insect) cells or vertebrate cells, e.g., *Xenopus laevis* oocytes or mammalian cells, in particular insect and mammalian cells. Examples of suitable mammalian cell lines are the COS (e.g., ATCC CRL 1650), BHK (e.g., ATCC CRL 1632, ATCC CCL 10) or CHO (e.g., ATCC CCL 61) cell lines.

The host cell may be a mammalian basophilic cell or mammalian hybrid cell. The mammalian basophilic cell may be human, guinea pig, rabbit or rat basophilic cells. In a specific embodiment, the mammalian basophilic cell is a rat basophilic cell. In a most specific embodiment, the rat basophilic cell may be an RBL-1 cell having the identifying characteristics of ATCC CRL-1378 or RBL-2H3 cell having the identifying characteristics of ATCC CRL 2256.

Methods for transfecting mammalian cells and expressing DNA sequences introduced in the cells are described in e.g., Kaufman and Sharp, 1982, J. Mol. Biol. 159:601-621; Southern and Berg, 1982, J. Mol. Appl. Genet. 1:327-341; Loyter et al., 1982, Proc. Natl. Acad. Sci. USA 79:422-426; Wigler et al., 1978, Cell 14:725; Corsaro and Pearson, 1981, Somatic Cell Genetics 7:603, Graham and van der Eb, 1973, Virology 52:456; Fraley et al., 1980, JBC 225:10431; Capecchi, 1980, Cell 22:479; Wiberg et al., 1983,NAR 11:7287; and Neumann et al., 1982, EMBO J. 1:841-845. In a specific embodiment, the mammalian basophilic cell is transfected with DNA encoding HBP using an electroporation apparatus.

The medium used to culture the cells may be any conventional medium suitable for growing mammalian cells, such as a serum-containing or serum-free medium containing appropriate supplements, or a suitable medium for growing mammalian cells. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in catalogues of the American Type Culture Collection). The cells are then screened for antibiotic resistance. Subsequently, the selected clones are subsequently assayed for HBP activity using assays known in the art such as a chemotaxis assay and assaying for cytokine release from monocytes (see, for example, Rasmussen et al., 1996, FEBS Lett. 390:109-112).

Alternatively, the host cell may be a hybrid mammalian cell. A myeloma line e.g., mouse, rat, human) is transfected with DNA encoding HBP using the procedures described above. It may be subsequently be fused with a mammalian cell expressing an acidic proteoglycan such as a mammalian basophilic cell or mast cell using the following procedures. In one embodiment the parental cells are mixed in culture media such as RPMI-1640 and exposed to a chemical fusion agent such a polyethylene glycol (see, for example, Gefter et al., 1997, Somat. Cell Genet. 3:231-236). The fusion agent is subsequently diluted out and the cells are incubated in media and HAT. Selected clones are subsequently assayed for HBP activity as described above.

Alternatively, two parental cells may be fused by electrofusion. Membrane contact between cells are achieved by a non-uniform alternating field that leads to dielectrophoresis and cell chain formation. Fusion is then triggered by the injection of a field pulse that is strong

enough to induce reversible breakdown in the membrane contact zone (see, for example, Okada et al., 1984, Biomed. Res. 5:511-566). Alternatively cell fusion may be induced by Sendai virus (see, for example, Wainberg et al., 1973, J. Cell Biol. 57:388-396).

The host cell may be a unicellular pathogen, e.g., a prokaryote, or a non-unicellular pathogen, e.g., a eukaryote. Useful unicellular cells are bacterial cells such as gram positive bacteria including, but not limited to, a *Bacillus* cell, e.g., *Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus brevis, Bacillus circulans, Bacillus coagulans, Bacillus lautus, Bacillus lentus, Bacillus licheniformis, Bacillus megaterium, Bacillus stearothermophilus, Bacillus subtilis*, and *Bacillus thuringiensis*; or a Streptomyces cell, e.g., *Streptomyces lividans* or *Streptomyces murinus*, or gram negative bacteria such as *E. coli* and *Pseudomonas* sp. In a preferred embodiment, the bacterial host cell is a *Bacillus lentus, Bacillus licheniformis, Bacillus stearothermophilus* or *Bacillus subtilis* cell. The transformation of a bacterial host cell may, for instance, be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, Molecular General Genetics 168:111-115), by using competent cells (see, e.g., Young and Spizizin, 1961, Journal of Bacteriology 81:823-829, or Dubnar and Davidoff Abelson, 1971, Journal of Molecular Biology 56:209-221), by electroporation (see, e.g., Shigekawa and Dower, 1988, Biotechniques 6:742-751), or by conjugation (see, e.g., Koehler and Thorne, 1987, Journal of Bacteriology 169:5771-5278).

The host cell may be a fungal cell. "Fungi" as used herein includes the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota (as defined by Hawksworth et al., In, Ainsworth and Bisby's Dictionary of The Fungi, 8th edition, 1995, CAB International, University Press, Cambridge, UK) as well as the Oomycota (as cited in Hawksworth et al., 1995, supra, page 171) and all mitosporic fungi (Hawksworth et al., 1995, supra). Representative groups of Ascomycota include, e.g., Neurospora, Eupenicillium (=Penicillium), Emericella (=Aspergillus), Eurotium (=Aspergillus), and the true yeasts listed above. Examples of Basidiomycota include mushrooms, rusts, and smuts. Representative groups of Chytridiomycota include, e.g., Allomyces, Blastocladiella, Coelomomyces, and aquatic fungi. Representative groups of Oomycota include, e.g., Saprolegniomycetous aquatic fungi (water molds) such as Achlya. Examples of mitosporic fungi include Aspergillus, Penicillium, Candida, and Alternaria. Representative groups of Zygomycota include, e.g., Rhizopus and Mucor.

In a preferred embodiment, the fungal host cell is a yeast cell. "Yeast" as used herein includes ascosporogenous yeast (Endomycetales), basidiosporogenous yeast, and yeast belonging to the Fungi Imperfecti (Blastomycetes). The ascosporogenous yeasts are divided into the families Spermophthoraceae and Saccharomycetaceae. The latter is comprised of four subfamilies, Schizosaccharomycoideae (e.g., genus Schizosaccharomyces), Nadsonioideae, Lipomycoideae, and Saccharomycoideae (e.g., genera Pichia, Kluyveromyces and Saccharomyces). The basidiosporogenous yeasts include the genera Leucosporidim, Rhodosporidium, Sporidiobolus, Filobasidium, and Filobasidiella. Yeast belonging to the Fungi Imperfecti are divided into two families, Sporobolomycetaceae (e.g., genera Sorobolomyces and Bullera) and Cryptococcaceae (e.g., genus Candida). Since the classification of yeast may change in the future, for the purposes of this invention, yeast shall be defined as described in Biology and Activities of Yeast (Skinner, F.A., Passmore, S.M., and Davenport, R.R., eds, Soc. App. Bacteriol. Symposium Series No. 9, 1980). The biology of yeast and manipulation of yeast genetics are well known in the art (see, e.g., Biochemistry and Genetics of Yeast, Bacil, M., Horecker, B.J., and Stopani, A.O.M., editors, 2nd edition, 1987; The Yeasts, Rose, A.H., and Harrison, J.S., editors, 2nd edition, 1987; and The Molecular Biology of the Yeast Saccharomyces, Strathern et al., editors, 1981).

In a more preferred embodiment, the yeast host cell is a cell of a species of Candida, Kluyveromyces, Saccharomyces, Schizosaccharomyces, Pichia, or Yarrowia. In a most preferred embodiment, the yeast host cell is a Saccharomyces carlsbergensis, Saccharomyces cerevisiae, Saccharomyces diastaticus, Saccharomyces douglasii, Saccharomyces kluyveri, Saccharomyces norbensis or Saccharomyces oviformis cell. In another most preferred embodiment, the yeast host cell is a Kluyveromyces lactis cell. In another most preferred embodiment, the yeast host cell is a Yarrowia lipolytica cell.

The medium used to culture the cells may be any conventional medium suitable for growing mammalian cells, such as a serum-containing or serum-free medium containing appropriate supplements, or a suitable medium for growing insect, yeast or fungal cells. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g,. in catalogues of the American Type Culture Collection).

The HBP produced by the cells may then be recovered from the culture medium by conventional procedures including separating the host cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g., ammonium sulphate, purification by a variety of chromatographic procedures, e.g., ion exchange chromatography, affinity chromatography, or the like. The recombinant host cells may also produce an acid proteoglycan such as heparin sulfate. To obtain active HBP, the acid proteoglycan will need to be removed. This may be accomplished using a series of separation methods, ie., precipitation or column chromatography, such as reverse phase HPLC, HIC, SEC, IEC and affinity based techniques. The separation method may be combined with other treatments like increasing salt concentration, by change in the pH and by other means that reduce interactions between the acidic proteoglycan and HBP.

The recombinant host cells may also produce an acid proteoglycan such as heparin sulfate. To obtain active HBP, the acid proteoglycan will need to be removed.

COMPOSITIONS

In the pharmaceutical composition used in the method of the present invention, the HBP may be formulated by any of the established methods of formulating pharmaceutical compositions, e.g. as described in Remington's Pharmaceutical Sciences, 1985. The composition may typically be in a form suited for local or systemic injection or infusion and may, as such, be formulated with sterile water or an isotonic saline or glucose solution. The compositions may be sterilized by conventional sterilization techniques which are well known in the art. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with the sterile aqueous solution prior to administration. The composition may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as buffering agents, tonicity adjusting agents and the like, for instance sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, etc. The concentration of HBP may vary widely, i.e. from less than about 0.5%, such as from 1%, to as much as 15-20% by weight. A unit dosage of the composition may typically contain from about 10 mg to about 1 g of HBP.

HBP or pharmaceutically active fragments thereof are administered topically or by intravenous injection. Dosages will be prescribed by the physician according to the particular condition and the particular individual to be treated. Dosages and frequency is carefully adapted and adjusted according to parameters determined by the physician in charge. A preferred administration route may be e.g. injections intraperitoneally. Intravenous intraperitoneal injections of HBP may be given per 24 hours in the range of from 0.1-100 mg, especially 0.1-20 mg, in particular 0.1-10 mg per kg body weight. The dose may be given 1-4 times per 24 hours or administered continuously through a catheter.

Compositions used in the present invention may additionally comprise a mitochondrial matrix targeting protein. In a specific embodiment, the mitochondrial matrix targeting protein has a molecular weight of about 33 kD, binds to H- but not L-kininogen. In a more specific embodiment, the mitochondrial matrix targeting protein has an N-terminal amino acid sequence depicted in SEQ ID NO:15, the 32 N-terminal amino acid sequence of p33 (Ghebrehiwet et al., 1994, J. Exp. Med. 179: 1809):

MLPLLRCVPRVLGSSVAGLRAAAPASPFRQLL (SEQ ID NO:15)

In a most specific embodiment the mitochondrial matrix targetting protein is the zinc dependent p33 protein depicted in SEQ ID NO:16:

MLPLLRCVPRVLGSSVAGLRAAAPASPFRQLLQPAPRLCTRPFGLLSVRAGSERRPGL
LRPRGPCACGCGCGSLHTDGDKAFVDFLSDEIKEERKIQKHKTLPKMSGGWELELNG
TEAKLVRKVAGEKITVTFNINNSIPPTFDGEEEPSQGQKVEEQEPELTSTPNFVVEVIK
NDDGKKALVLDCHYPEDEVGQEDEAESDIFSIRESFQSTGSEWKDTNYTLNTDSLDW
ALYDHLMDFLADRGVDNTFADELVELSTALEHQEYITFLEDLKSFVKSQ (SEQ ID NO:16)

Compositions used in the methods of the present invention are contemplated to be of use in treating or preventing disorders caused by apoptosis. These include but are not limited to

HIV, neurodegenerative or neuromuscular diseases, ischemic stroke, anoxia, ischemia/reperfusion damage and intoxication septic shock.

In a specific embodiment, said compositions may be used to prevent the destruction of beta cells in the Islets of Langerhans in the pancreas. Damage to such beta cells leads to diabetes mellitus.

EXAMPLES

Example 1: Treatment of Beta Cells with HBP

Production of HBP

HBP is obtained from RBL-1 cells using the procedure described in application serial no. PCT/DK98/00275. The procedure is summarized below.

Materials

The vectors pBlueBacIII and pcDNA3 are obtained from Invitrogen. All primers and oligos are synthesized on an Applied Biosystems Model 394 DNA synthesizer. Restriction enzymes are obtained from New England Biolabs. Pfu polymerase, used in PCR reactions is obtained from Stratagene. RBL-1 cells (ATCC CRL-1378) and RBL-2H3 cells (ATCC CRL-2256) are obtained from American Type Culture Collection (ATCC) in Manassas, VA. Cells are grown as recommended by the supplier or in RPMI 1640 culture medium (Gibco, Life Technologies) supplemented with 10% heat inactivated gamma- irradiated FCS (origin: NZ, Gibco, Life Technologies) or fetal calf serum (FCS) North American origin from HyClone or BioWhittaker. Cells are grown in 5% CO_2 at 37°C in an 80% humidified atmosphere. Exponentially growing cells are used in all experiments.

C nstruction of Expression Vect rs

A 770 bp BamHI-HindIII fragment is constructed using PCR technology from a human bone marrow DNA library (Clontech) based on the human HBP amino acid sequence (Flodgaard et

al., 1991, Eur. J. Biochem. 197:535-547) and the CAP 37/azurocidin DNA sequence (Morgan et al., 1991, J. Immunol. 147:3210-3214 and Almeida et al., 1991, Biochem. Biophys. Res. Commun. 177:688-695). This fragment contains the entire coding region of HBP, including a 19-residue signal peptide, a 7 amino acid pro-peptide, a mature part of 22 amino acids, and a 3 amino acid C-terminal extension. The fragment is inserted into pBlue-BacIII resulting in the plasmid pSX556. For deletion of the pro-region, an oligonucleotide linker of 99 bp, covering the signal peptide and the first 4 amino acids of mature HBP (from BamHI to EagI) is substituted for the original BamHI-EagI fragment in pSX556 giving rise to pSX559.

For expression of these two cDNA sequences in RBL-1 cells, pSX556 and pSX559 described above are used as templates in PCR reactions using the primers
PBRa 246 (5'-CCGGGGATCCAACTAGGCTGGCCCCGGTCCCGG-3') (SEQ ID NO:13)
PBRa247 (5'-CCGGGGATCCGATGACCCGGCTGACAGTCCTGG-3') (SEQ ID NO:14)
with a Pfu polymerase according to manufacturer's instructions (Stratagene).

After *BamH*I cleavage of the PCR reaction products, the fragments are ligated in correct orientation into the mammalian expression vector pcDNA3 (Invitrogen), linearized with *BamH*I, resulting in two plasmids, pcDNA3-HBP and pcDNA3-HBP pro.

Transfection Procedures

Transfection is performed according to the following procedures. 25 μg of pcDNA3-HBP or pcDNA3-HBP pro is transfected into RBL-1 cells or RBL-2H3 cells (8×10^6 cells are transfected using a BioRad Electroporation Apparatus with electric settings 960 uF and 300V as described by Gullberg et al., 1994, J. Biol. Chem. 269:25219-25225 and Garwicz et al., 1995, J. Biol. Chem. 270:28413-28418, or are transfected using LipofecAmine (Gibco, Life Technologies) or Superfect (Qiagen) transfection reagents as recommended by the suppliers. Cells are grown in RPMI 1640 medium supplemented with 10% heat inactivated gamma irradiated FCS in 5% CO_2 at 37°C in an 80% humidified atmosphere. Geneticin (2 mg/ml) is added 48 hrs. post-transfection to select for recombinant clones.

Individual clones growing in the presence of geneticin are isolated and tested for HBP expression by ELISA. The HBP ELISA is a sandwich immunoassay using a monoclonal antibody as catcher and a polyclonal rabbit antibody conjugated to horseradish peroxidase as detector. Antibodies are prepared according to standard procedures by immunizing mice and rabbits with HBP purified from human buffy coat cells (Flodgaard et al., 1991, Eur. J. Biochem. 197:535-547). Specifically, each well is coated with 0.5 µg monoclonal anti-hHBP dissolved in 100 µl of PBS overnight. The coated wells are washed three times with a solution of 5% lactose, 0.5% Byco A 0.05% Tween 20 and 0.024% thiomersal. After the last washing, the plates are left to dry at room temperature upside-down on a piece of cloth. The coated plates ~~are rapped with staniol and can be stored up to three months.~~

Purified hHBP is used as reference preparation. A working dilution of 100 ng hHBP/ml is prepared in a BSA-EDTA buffer and stored in aliquots at -80°C for a maximum of two weeks. Serial dilution's containing 0; 0.3; 1; 4 and 12 ng hHBP/ml diluted in BSA-EDTA are made fresh and 100 µl are added to each well. hHBP samples are also diluted in BSA-EDTA buffer and all the samples are in-cubated agitated for 1 hour at room temperature. The wells are emptied and washed three times with phosphate buffered saline followed by the addition of 100 µl/well diluted (1:1000) Fab-peroxidase conjugated rabbit anti-hHBP, and incubated agitated for 1 hour at room temperature. Peroxidase activity is measured using 100 µl/well TMB perborate substrate solution, resulting in a color formation measurable photometrically at 450 nm. The reference curve is linear when the logarithm to the absorbance is plotted against the logarithm to the dose.

Clones with the most pronounced expression are chosen for further experiments, recloned and retested for expression levels. The highest HBP producers are selected and grown into mass culture or adapted to serum free or protein free medium.

Isolation of HBP

The isolation of HBP from RBL-1 cells is carried out essentially as described by Rasmussen et al., 1996, FEBS Lett. 390:109-112. The transfected and selected RBL-1 cells are initially filtered to remove any remaining cells and cell debris. The culture medium is subsequently

applied to a CM-Sepharose cation-exchange column (Pharmacia and Upjohn), previously equilibrated with 50 mM sodium phosphate, pH 7.3. Unbound and loosely bound materials are eluted with equilibration buffer until baseline is achieved measured by on-line UV detection at 280 nm. The column is then developed with a linear gradient from 0 to 1 M sodium chloride in equilibration buffer. HBP eluted with about 0.7 M sodium chloride and fractions are combined based on UV absorption. Pooled fractions are diluted with two volumes of distilled water and applied on a new CM-Sepharose column. Following equilibration HBP is step eluted with 1 M sodium chloride in equilibration buffer and fractions combined based on absorption at 280 nm. Highly concentrated and pure HBP is obtained by this procedure. Final purification is carried out on a Sephadex G-25 gel-filtration column (Pharmacia & Upjohn) equipped with a UV-flow cell and equilibrated and eluted with 0.02% trifluoroacetic acid. HBP is collected based on absorption at 280 nm. The gel filtration serves mainly as a buffer exchange step to produce a stable preparation of HBP that is kept at 4°C until use.

HBP Rescues Beta Cells from Cytokine Induced Apoptosis

The following protocol is used:

Day 0: Rat Insulinoma cells (RIN cells) are seeded in 200 μl medium with 10,000 cells per well. Day 1:After 24 hours the medium is discarded and new medium containing 0, 20 or 50 μg/ml medium of HBP is added to a total volume of 100 μl. Day 2: The medium in the wells without HBP is discarded and fresh medium containing 0 or 1500 units IL-1 beta and 20 μg/ml medium of HBP is added in a total volume of 200 μl. To the wells pretreated with HBP (cells not attached) 0 or 1500 units IL-1 Beta and 20 μg/ml medium of HBP is added in a total volume of 100 μl giving a total volume of 200 μl. Day 5: The NO content in the medium and the accumulation of insulin are measured. MTT assay (succinate dehydrogenase activity, a measure of apoptosis) is performed on the cells.

The results are shown in Figures 1 and 2. The numbers 1-6 depict the following: 1:Control (all media changed after preincub. with HBP, 200 μl new media with HBP and cytokines added; 2: as 1 but with 20 μg/ml HBP; 3: as 1 but with 50 μg/ml HBP; 4: Control (100 ml media (with HBP and 2x cytokines) added on top of 100 μl HBP preincubation; 5: as 4 but

with 20 μg/ml HBP; 6: as 4 but with 50 μg/ml HBP. It is evident from the results that treatment of beta cells with HBP results in a decreased apoptosis, but no effect on NO production.

Effect of HBP on Streptozotocin Treated Cells

Beta cells are pre-incubated with HBP or control vehicle for 24 hours before treatment with varying concentrations of the NO donor, Streptozotocin. After 1-2 days of incubation at standard conditions (37°C, 5% CO_2) cells are assayed for apoptosis. The cells pre-incubated with HBP show less or no apoptosis as compared to control incubations. Thus, HBP prevents destruction of the beta cells.

Beta cells are incubated with HBP and Streptozotocin or control vehicle and Streptozotocin for 24 hours. The cells incubated with HBP show less or no apoptosis as compared to control incubations. Thus, HBP prevents destruction of the beta cells.

Animal Studies

Wistar adult rats are pre-treated with a sustained release of HBP or control vehicle from a subcutaneously implanted mini osmotic pump on the back. 24 or 48 hours later, the rats are rendered diabetic by intraperitoneal injection of Streptozotocin in the tail. Blood glucose and urinary excretion of glucose and albumin are monitored for 2-3 weeks. The HBP treated rats show a significant lower frequency of diabetes symptoms than the controls. Finally, the animals are sacrificed and histological examination of the pancreas is performed. The HBP treated rats show no or little beta cell destruction.

Spontaneously diabetic NOD (nonobese diabetic) mice are treated with HBP or control vehicle for 1-2 months. The HBP treated mice show a significant lower frequency of diabetes symptoms than the controls. Finally, the animals are sacrificed and histological examination of the pancreas is performed. The HBP treated mice show no or little beta cell destruction.

Three month old obese Zucker rats and age-matched lean rats are treated for 1 month with HBP by subcutaneously infusion using mini-osmotic pumps delivering 0.05 mg/kg body

weight per 24 hours. After 1 month the animals are sacrificed and the pancreas assessed for damage of Islets of Langerhans by histological techniques known to the person skilled in the art.

Example 2: HBP is Internalized and Targeted to the Mitochondrial Compartment

Experimental procedures

Cell culture

Human umbilical vein endothelial cells (HUVEC) are isolated by digestion with collagenase (Worthington Biochemical, Freehold, NJ, USA) and cultured on gelatinized (Sigma Chemical Co., St. Louis, MO, USA) surfaces in the presence of fetal calf serum (ICN Biochemicals Inc., Costa Mesa, Ca, USA), calf serum (ICN Biochemicals Inc.) endothelial cell growth factor (Sigma), heparin (Sigma) and antibiotics (Gibco BRL, Paisley, Scotland) in M199 with Earle's salt (GIBCO) (Jaffe et al., 1973, J. Clin. Invest. 52:2745-2756 and Thornton et al., 1983, Science 222:623-625). The primary cultures are passed only once using trypsin-EDTA. The cells are used when expressing cobblestone morphology except for microscopical studies when subconfluent cells are used.

Wildtype CHO cells (CHO-K1) and the heparan sulfate deficient variant (pgsD-677) (Murphy Ulrich et al., 1988, J. Biol. Chem. 272:24363-243670). are grown in F12K Nutrient mixture with Kaighn's modification (GIBCO) supplemented with fetal calf serum and antibiotics.

White 96 well tissue culture plates, CulturePlates™ , Microscint-PS scintillation fluid and the microplate scintillation counter Topcount™ is obtained from Packard, Meriden, USA Standard 96 well tissue culture plates are obtained from Nunc, Denmark. Fetal calf serum (FCS) is obtained from Gibco BRL. Hydrogen peroxide is obtained from Merck. Formaldehyde and Triton X-100 is obtained from Sigma.

Antibodies and proteins

Recombinant human HBP is produced using a baculovirus expression system in Sf9 insect cells (BRL) and purified as described (Rasmussen et al., 1996, FEBS Lett. 390:109-112). The mouse monoclonal antibody (mab) 2F23C3 and rabbit antisera against recombinant HBP (anti-HBP) were affinity-purified. The antiserum against mitochondrial protein p33/gC1qR (anti-p33) was raised in rabbits and affinity-purified (Dedio et al., 1998, J. Immunol 160:3534-3542). The mouse mab 3G10 recognizing desaturated glucuronate reactive with heparitinase-digested heparan sulfate proteoglycan core proteins has been characterized previously (David et al., 1992, J. Cell Biol. 119:961-975). Texas red-conjugated goat anti-rabbit immunoglobulin IgG and goat anti-mouse IgG are from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA); peroxidase-conjugated antibodies against rabbit IgG from Bio-Rad (Richmond, CA, USA); and alkaline phosphatase-conjugated rabbit anti-mouse antibodies from Promega Corporation (Madison, WI, USA). Biotinylation of purified HBP is performed.

Metabolic labeling of endothelial cells

Endothelial cells are cultured in the presence of 100 mCi/ml $Na_2{}^{35}SO_4$ (specific activity) (Amersham International, Buckinghamshire, UK) or 50 mCi/ml ^3H leucine (Amersham) diluted in endothelial cell culture media. After 24 hours, the cells are washed 4 times with cold phosphate buffered saline (PBS, GIBCO) and lysed for 1 hour at 4°C on a shaker. The lysis buffer consisted of 1% Triton-X-100, 20 mM Tris-HCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM PMSF, 5 mM 1,10-phenanthroline, 4 mg/ml leupeptin, 4 mg/ml pepstatin A and 100 mg/ml aprotonin at pH 8.0. The lysate is centrifuged at 10,000 g for 30 minutes at 4°C and the pellet is discarded.

Affinity chromatography on HBP Sepharose

The endothelial cell lysate, containing 0.2 M NaCl, is incubated end over end with DEAE Sepharose Fast Flow (Pharmacia Biotech AB, Uppsala, Sweden) pre-equilibrated with 0.2 M NaCl, 20 mM Tris-HCl, 0.1% Triton-X-100 and 1 mM PMSF at pH 8.0. After 1 h at 4°C the DEAE-Sepharose is washed with 10 gel volumes of 0.2 M NaCl, 50 mM Na-acetate, 0.1% Triton-X-100, 1 mM PMSF at pH 4.0 and then eluted with 1 M NaCl in buffer A (20 mM Tris HCl, 0.1% Triton-X-100 and 1 mM PMSF at pH 7.4).

Biotinylated HBP (5 mg) is bound to 2.5 ml streptavidin-agarose (Sigma). Uncoupled agarose is used as a control column. Successful coupling of HBP to the column is confirmed by SDS PAGE (data not shown) . Equal gel volumes of the HBP- and control agarose are equilibrated at 4°C with buffer A containing 150 mM NaCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$. The material eluted from the DEAE-Sepharose is dialyzed against equilibration buffer, incubated end over end with the control column for 2 h at 4°C followed by the HBP-column for 2 h at 4°C. The HBP- and control columns are washed with 10 gel volumes of equilibration buffer and then eluted with 5 gel volumes each of a discontinuous NaCl gradient ranging from 250 to 1000 mM NaCl in buffer A. Samples from eluted material are separated by 4-16% SDS-PAGE under reducing or non-reducing conditions. Analysis of radioactive material is done on gels exposed to X-ray film or to Fuji Imaging plates (BioImaging Analyzer Bas2000, Fuji Photo Film Co., LTD, Tokyo, Japan). When the samples contained ^{3}H-leucine-labeled material, gels are treated with 1.3 M Na-salicylate in 5 mM NaH_2PO_4 at pH 7 (Chamberlain, 1979, Anal. Biochem. 98:132-135) prior to exposure to X-ray film.

Enzymatic and Chemical Digestion of Proteoglycans

Radiolabeled HBP-binding material eluted from the HBP-streptavidin agarose is treated over night at room temperature with either 60 mU/ml CABC (Sigma), 4 U/ml heparinase III (Sigma), or a combination of both in 50 mM Tris-HCl, 0.1 M NaCl at pH 7.3. In order to cleave heparan sulfate, the samples are treated with HNO_2 at pH 1.5 for 10 min at room temperature (Shively and Conrad, 1976, Biochemistry 15:3932-3942). Proteoglycans can be separated based on their size in agarose gels where they run as discrete units rather than as a broad smear (Bjornsson, 1993, Anal. Chem. 210:282-291 and Bjornsson, 1993, Anal. Chem. 210:292 298). Some treated samples are separated on 1.2 % agarose gels whereas others are separated on 4-16% SDS-PAGE. The dried gels are exposed to Fuji Imaging plates.

Western blotting of proteoglycans

The HBP-affinity purified material is concentrated on 100 ml DEAE-Trisacryl columns (BioSepra S.A., Villeneuve la Garenne Cedex, France) and eluted with buffer A containing 1 M NaCl (5 x 50 ml). The concentrated samples and the sample from the original endothelial

cell lysate are doubly digested with 0.5 U/ml CABC (Seikagaku Kogyo, Tokyo, Japan) and 10 mU/ml heparitinase (Seikagaku) in 100 mM NaCl, 1 mM $CaCl_2$, 0.1% Triton-X-100, 50 mM 6-amino-hexanoic acid, 20 mg/ml leupeptin, 2.5 mg/ml pepstatin A, 1 mM PMSF and 50 mM HEPES at pH 7.0, for 3 hours at 37°C. After separation under non-reducing conditions by 20% SDS-PAGE, materials are transferred onto Zeta-Probe membranes (Bio-Rad laboratories, Hercules, CA, USA) at 70 V, 0.5 mA for 4 hours at 4°C. The membranes are blocked in 0.5% casein in PBS (buffer B) containing 0.6 M NaCl over night at 4°C followed by incubation for 1 hour at room temperature with mouse mAb 3G10, diluted in buffer B containing 0.15 mM NaCl. After two washes with buffer B containing 0.6 M NaCl, the membranes are washed ~~once in buffer B containing 0.15 M NaCl and then incubated with rabbit anti-mouse IgG~~ conjugated with alkaline phosphatase diluted 1:5000 in buffer B containing 0.15 M NaCl. After two washes, bound antibodies are detected using the CSPD detection system (Tropix, Bedford, MA, USA) according to the manufacturer.

Western blotting of p33

Protein samples eluted from the HBP-coupled column are separated by 12.5 % SDS-PAGE and transferred onto nitrocellulose membranes for 30 min at 100 mA. The membranes are blocked with 50 mM KH_2PO_4, 0.2 M NaCl, containing 5% w/v dry milk powder and 0.05% w/v Tween 20 at pH 7.4 (buffer C). Transferred proteins are incubated with primary antibodies (anti-rp33, anti-CDR31, anti-MBP, anti-HBP) diluted to 1 mg/ml in buffer C. Bound primary antibodies are detected by a peroxidase-conjugated secondary antibody against rabbit IgG followed by the ECL (Amersham) detection method.

Indirect ELISA

Microtiter plates are coated overnight at room temperature with HBP, H-kininogen or a control peptide KLH (1 mg/ml each) in 100 mM sodium acetate and 100 mM NaCl at pH 5.5 (Herwald et al., op. Cit.). Binding of the recombinant fusion protein rp33 or the fusion partner MBP (starting concentration 2 mg/ml, two fold dilutions) to the immobilized proteins is detected by a-MBP (1:2500 v/v) followed by incubation with a peroxidase-conjugated secondary antibody directed against rabbit IgG (1:3000 v/v) (Herwald et al., op. cit). The incubation steps are done in a buffer containing 50 mM KH_2PO_4, 0.2 M NaCl, containing 2% w/v bovine

serum albumin (BSA) and 0.05% w/v Tween 20 at pH 7.4. For visualization, a substrate solution of 0.15% w/v diammonium 2,2-azino-bis-(3-ethyl-2,3-dihydrobenzthiazoline-6-sulfonate), (ABTS), 0.012 % H_2O_2 in 100 mM citric acid, pH 4.5 is applied for 30 min, and the change in absorbance is determined at 405 nm.

Measurement of affinity between HBP and p33

Specific interactions between HBP and rp33 are studied using surface plasmon resonance spectroscopy (BIAlite, Pharmacia, Freiburg, Germany). HBP is coupled to a CM5 sensor chip according to the manufacturer's instructions. MBP and rp33 are dissolved in HEPES-Tyrodes buffer in the presence or in the absence of 50 µM Zn^{2+} in two fold serial dilution, with a starting concentration of 100 µg/ml. To follow the association of rp33 and MBP to coupled HBP, 30 µl of each protein sample is applied using a flow rate of 10 ml/min. After 3 min, the chip is washed with PBS to follow dissociation for 3 min. The chip is regenerated by washing with 30 mM HCl. To calculate the dissociation constant, the BIAevaluation 2.0 program (Pharmacia, Freiburg, Germany) is used.

FACS-analysis

Endothelial cells grown to confluency in 12-well plates are washed once in M199 with Hank's solution (GIBCO) and then incubated with unconjugated recombinant HBP (50 µg/ml) diluted in the same media for various periods of time at 37°C. The cells are washed twice with 0.5% human serum albumin (Calbiochem, La Jolla, CA, USA) in PBS and once with Ca^{2-} and Mg^{2+} free PBS. Cell dissociation solution (500 µl; Sigma) is added, the cells are placed at 37°C for 15 min and then harvested using a cell scraper. After fixation in 1% formaldehyde for a minimum of 1 hour at room temperature, the cells are incubated with a mouse mAb against HBP (25 µg/ml) in PBS/0.02% azide containing 1% heat-inactivated human serum or the same solution containing 1% saponin (Sigma) and 0.0125% digitonin (Sigma). Next, the cells are incubated with a FITC-conjugated secondary goat-anti-mouse antibody diluted 1:100. The cells (5,000/experiment) are analyzed on a FACSort (Becton Dickinson, Palo Alto, CA, USA) using a FACStation with Cellquest software. The control cells are incubated with

either primary or both primary and secondary antibodies but not with HBP. The mean fluorescence intensity (MFI) is calculated on channel values. Results are given as means SD.

In some experiments, the endothelial cells are pretreated with NH_4Cl (50 mM), cytochalasin D (1 μM) or cycloheximide (1 nM) before addition of HBP (50 μg/ml). The various substances are present throughout the 30 min incubation with HBP. In other experiments, HBP is incubated with heparin (100 μg/ml) prior to addition of the HBP-heparin mixture to the cells. The binding and internalization of HBP to endothelial cells is also investigated at 4°C. Wildtype (CHO-K1) and heparan sulfate proteoglycan deficient CHO cells (pgsD-677) (Murphy-Ulrich et al., op cit.) are treated as described above for endothelial cells and investigated for internalization of HBP.

Confocal laser microscopy

Endothelial cells grown overnight on microscopic slides are incubated with 50 μg/ml FITC labeled or unconjugated HBP in M199 with Hank's solution (GIBCO) for various periods of time. After a brief wash with PBS, the endothelial cells are fixed in 4% formaldehyde for 1 hour. Cells are washed with 100 mM glycine for 1 hour and permeabilized with cold methanol for 10 min. Cells are incubated with 1% BSA in PBS prior to incubation with antibodies. The staining steps are performed so as to avoid crossreactions from secondary reagents.

When FITC-conjugated HBP is used, the cells are incubated up to 24 hours, fixed in formaldehyde and stained for p33. After 30 min incubation with anti-rp33 (10 μg/ml), the cells are washed and fixed with a Texas Red-conjugated goat-anti-rabbit IgG. The slides are equilibrated and mounted with SlowFade Antifade (Molecular Probes, Leiden, The Netherlands) according to manufacturer's instructions. The cells are analyzed using a Zeiss LSM 310 (Laser Scan Microscope, Oberkochen, Germany). A 590 nm filter is used to prevent interference of emitted light from the green to the red signal. Horizontal sections of doublestained cells are taken and investigated for colocalization. Colocalization resulted in a color shift from green and red to yellow/orange. In some experiments, the signal from the green channel is subtracted from the red channel thereby yielding black areas representing colocalization.

When unconjugated HBP is used, the fixed cells are incubated with biotinylated rabbit-anti human-HBP antibody (50 mg/ml) for 30 min followed by FITC-conjugated streptavidin (10 mg/ml). Next the cells are incubated with a mouse mAb specific for human mitochondria (mAb 1273) diluted 1:50 followed by incubation with Texas red-conjugated goat-anti-mouse IgG. The cells are mounted and investigated for colocalization as described above.

Cell Fractionation

Confluent endothelial cells grown in five 12-well plates were washed once in M199 with ~~Hank's solution (GIBCO). Each plate was incubated with non-conjugated recombinant~~ wildtype HBP (25 mg/ml) in 5 ml of the same buffer for 24 h at 37°C. Cells are washed once with Ca^{2+} and Mg^{2+} free PBS, and scraped from the plates. After centrifugation (800 x g, 10 min) cells are resuspended in 2.5 ml of 50 mM phosphate, pH 7.4, 0.28 M sucrose, 100 μg/ml phenylmethanesulfonyl fluoride, 1 μg/ml aprotinin, 0.5 μg/ml leupeptin, 1 μg/ml pepstatin A, 3.6 μg/ml trans-epoxylsuccinyl-L-leucylamido-(4-guanidino)butane. Washed cells are pressurized with N_2 for 5 min at 350 psi at 4°C and the cavitate is collected. The homogenate is centrifuged at 800 x g for 10 min and the nuclear pellet P1 is discarded. The supernatant (S1) is further analyzed as described earlier Briefly, supernatant S1 is centrifuged at 20,000 x g for 20 min and the resulting supernatant S2 is collected. The membrane pellet P2 is washed in 5 ml of 50 mM phosphate buffer containing proteinase inhibitors and centrifuged again. Washed pellet, P2, is resuspended in 1 ml of phosphate buffer containing 12% (w/v) sucrose and layered on top of a 33% (w/v) sucrose cushion (10 ml) followed by centrifugation carried out at 100,000 x g for 3 h. The pellet P3 (vesicular fraction) was resuspended in phosphate buffer. The membranes at the gradient interface were collected, diluted with 30 ml of phosphate buffer and centrifuged (30,000 x g, 45 min). Pellet P4 (membrane fraction) is resupended in phosphate buffer. Centrifugation (100,000 x g, 3 h) of supernatant S2 gave pellet P3 (microsomal fraction) and supernatant S3 (cytosolic fraction). All treatments are performed at 4°C.

Induction of Apoptosis in HUVEC Cells

HUVEC cells are retrieved and subcultured. Cells in passage #1 are trypsinized and seeded (10,000 cells/well in 100 µl complete growth medium) in 96-well CulturePlates, precoated with gelatine. The cells are cultured overnight to obtain confluence. As a visual control, cells are seeded in parallel in a standard 96-well tissue culture plate (Nunc).

After culturing overnight, the medium is changed to M199 + 10% FCS, hHBP is added to final concentrations 0, 10 or 50 µg/ml and the cells are incubated for 24 hours. After the preincubation period, apoptosis is induced by simultaneously washing away hHBP and changing the medium to either M199 + 10% FCS or M199 without additives. The cells are subsequently incubated for 24 hours. The DNA fragmentation is measured by the TUNEL as described in the kit sold by PharMingen method with a few modifications. At the end of the assay, culture medium is carefully removed and the cells are fixe by adding 200 µl 10% formaldehyde buffered in PBS (20 mM phosphate, 150 mM NaCl, pH 7.4) 0 for 30 min. at room temperature. The cells are washed once with PBS and permeabilized for 5 min. with 100 µl of a mix of 0.1% sodium citrate and 0.1 % Triton X-100 at room temperature, followed by a wash with PBS. The TUNEL reaction is initiated by adding 50 µl of TUNEL reaction mix (5 U Tdt enzyme and 0.3 µl [^{32}P]dCTP/well in 200 mM sodium cacodylate, 25 mM Tris-HCl, 1 mM $CoCl_2$, 0.25 g/l BSA, pH 6.6), the plate is incubated at 37°C for 1 hr. to determine the background level, wells in parallel are incubated with reaction mix without Tdt enzyme. The reaction is terminated by carefully removing the TUNEL reaction mix and washing the cells twice with PBS. Subsequently, the plate is completely dried under vacuum, 200 µl of Microscint-PS scintillation fluid is added, the plate is sealed and counted in a microplate scintillation counter (Packard TopCount). The degree of labelling is determined by subtracting the background level from the values obtained in the samples containing Tdt enzyme.

Effect of HBP on Hydrogen Peroxide Treated HUVEC Cells

HUVEC cells are treated with medium (control) and medium containing hydrogen peroxide as indicated for 18 hours. Apoptosis is determined as described above.

Results

Release of HBP from activated human neutrophils

HBP is a protein that is almost exclusively synthesized and stored in polymorphonuclear (PMN) leukocytes. To demonstrate that HBP can be released from these cells, PMN leukocytes are stimulated from human plasma with increasing concentrations of phorbol myristate acetate (PMA) or f-Met-Leu-Phe (fMLP) in the presence or absence of human umbilical vein endothelial cells (HUVECs). The agonist-triggered release of HBP was followed by sandwich ELISA and is exemplified for PMA (Figure 3A) and fMLP (Figure 3B). PMA and fMLP does induce HBP secretion from PMNs in a dose-dependent manner. The presence of endothelial cells further increases HBP secretion. Hence human PMNs efficiently secrete HBP upon stimulation by PMA or fMLP in proximity to HUVECs.

Affinity purification of HBP binding sites

Endothelial cells are metabolically labeled with $Na_2[^{35}S]SO_4$ to allow incorporation into the glycosaminoglycan chains of proteoglycans. Cells are lysed, the total cellular lysate was applied to DEAE-Sepharose, and the eluted material was affinity-purified on HBP covalently bound to agarose. The radiolabeled material was eluted from the HBP-column by a step gradient of 250 to 500 mM NaCl, and the resultant fractions were analyzed by SDS-PAGE (Figure 4A, left panel). The eluted material appears as a broad smear of bands covering a molecular weight range of approximately 40 kDA to > 400 kDa as often found for proteoglycans whereas minor amounts of unspecifically bound material was eluted from the uncoupled matrix (Figure 4A, right panel). Hence, a heterogeneous population of $[^{35}S]$-labeled molecules sticks to the HBP-Sepharose which may - at least in part - represent proteoglycans; these are referred to HBP binding sites.

Glucosidase treatment of HBP-binding proteoglycans

Radioactively labeled material from the HBP column was treated with chondroitinase$_{ABC}$ (C_{ABC}) to remove side chains of chondroitin sulfate and dermatan sulfate, or with HNO_2 to selectively destroy heparan sulfate side chains. The resultant mixtures were electrophoretically separated on agarose gels (Figure 4B). The $^{35}SO_4$-labeled HBP-binding sites were sensitive to

the treatment with C_{ABC} (left panel) or HNO_2 (right panel) indicating that proteoglycans containing chondroitin sulfate, dermatan sulfate and/or heparan sulfate side chains form part of the isolated HBP binding sites. Treatment with heparinase III which cleaves heparan sulfate side chains partially digests the HBP binding sites whereras the combined action of heparinase III and C_{ABC} results in a complete digestion. These data further indicate that proteoglycans are involved in HBP binding.

Identification of HBP-binding proteoglycans

~~Notably, endothelial cells express six major types of proteoglycans containing glycosamino-~~glycan of the heparan sulfate type, i.e. perlecan, glypican, and syndecan-1, -2, -3 and –4. Affinity-purified HBP binding sites were treated by double digestion with heparinase III and C_{ABC} to completely remove their heparan side chains (see above). The resultant cleavage products are separated by SDS PAGE, and subjected to Western blot analysis using the mouse monoclonal antibody mAb 3G10 that recognizes desaturated glucuronate, a neo-epitope of heparan sulfate proteoglycans exposed upon heparinase treatment (Figure 4C). For comparison, proteoglycans of total HUVEC lysates are run in parallel and identified by the relative molecular masses of their core proteins: syndecan-4 (35 K), syndecan-2 (48 K), glypican (64 K), syndecan-1 (90 K), syndecan-3 (125 K), and perlecan (> 200 K), Figure 4 (right panel, from bottom to top). All the heparan sulfate-containing proteoglycans present in total HUVEC lysates are also present in the HBP-binding fraction (left panel) indicating that they represent docking sites for HBP.

Inhibition of HBP internalization

To further characterize the HBP internalization process, FACS analyses is performed on HUVECs that are preincubated with 50 µg/ml of HBP for 0.5 h followed by extensive washes to remove the free ligand (Figure 5A). Permeabilized HUVECs showed a significantly higher mean fluorescence index (MFI) than intact cells suggesting that a significant fraction of the exogenously applied HBP had entered the cells. Incubation in the absence of HBP resulted in a MFI similar to that of intact cells indicating that the ligand had been effectively removed. Preincubation of the cells with 100 µg/ml of heparin effectively prevented uptake of HBP.

possibly due to competition with HBP for binding sites on heparan sulfate-containing proteoglycans. Incubation at 4°C significantly decreased HBP internalization for permeabilized HUVECs but not for non-permeabilized cells demonstrating that HBP internalization is an energy-dependent process. Addition of NH4Cl which has been shown to interfere with ligand release from internalized receptors thus leading to destruction rather than recirculation of internalized receptors (Gekle et al., 1995, Am. J. Physiol. 268:F899-906 and Rao et al., 1983, FEBS Lett. 160:213-216) drastically reduces HBP internalization. Cytochalasin D, an inhibitor of actin filament polymerization (Cooper, 1987, J. Cell Biol 105:1473-1478), lowers HBP internalization by 40.6%, whereas cycloheximide, an inhibitor of protein synthesis (Ennis, 1964, FEBS Lett. 399:255-258), diminishes HBP internalization. Colchicine, an inhibitor of microtubulus assembly (Olmstead and Borisy, 1973, Ann. Rev. Biochem. 42:507-540) decreases HBP internalization by 21%. Together these findings point to the fact that HBP internalization is an active process that requires an intact and functional cytoskeleton.

Role of proteglycans in HBP internalization

To further pinpoint the potential role of proteoglycans in HBP internalization, heparan sulfate proteoglycan-deficient chinese hamster ovary (CHO) cells, pgsD-677 (Murphy-Ulrich et al., 1988, J. Biol. Chem. 272:24363-243670) and the corresponding wild-type CHO cells are used to study HBP internalization by FACS analysis. The cells are incubated with 50 μg/ml HBP for various periods of time, and HBP content is analyzed in fixed and permeabilized cells (Figure 5B). Progressive internalization of exogenously added HBP was seen over 3 hours with the wild-type CHO cells. Heparan sulfate-deficient cells also internalized HBP though at a considerably lower efficiency: internalization was decreased by 33% (30 min), 38% (1h) and 57% (3h), respectively in pgsD-677 cells compared to wild-type CHO-K1 (100% at each time point). This finding suggests that heparan sulfate-type proteoglycans are involved in HBP internalization though other sites capable of internalizing HBP must exist, e.g. chondroitin sulfate-containing proteoglycans known to be overexposed by pgsD-677 cells (Murphy-Ullrich, 1988, op. cit.). Therefore we treated pgsD-677 cells with CABC for 30 min prior to incubation with HBP. The CABC treatment further decreased HBP uptake by 13% (30 min) whereas it had no significant effect on wild-type CHO-K1 cells. These findings lend support to our notion that both heparan sulfate- and chondroitin sulfate-type of proteoglycans are critical to

HBP internalization though other sites may be involved in HBP internalization by CHO cells. They also demonstrate that cells other than HUVECs can specifically bind and internalize HBP.

Subcellular fractionation of HBP-treated HUVECs

The subcellular localization of HBP is examined by fractionation of homogenates of HUVEC cells that had been preincubated with unlabelled HBP for 24 h at 37°C. Equal amounts of protein from the various cell fractions are subjected to Western blotting using anti-HBP (Figure 7, upper panel). A major 35 kDa band and a minor 29 kDa band were present in the vesicular (lane 2) and microsomal fractions (lane 4) but not in the cytosolic (lane 1) or membrane fractions (lane 3). Note that, the majority of internalized HBP retain the molecular mass of the native protein (35 K) suggesting that it is still in the intact form. Control HUVECs kept with buffer alone failed to reveal specific immunoreactive bands . An endogenous protein, p33/gC1qR which has been demonstrated in the vesicular fraction of HUVECs (Dedio et al., 1996, FEBS Lett.399:255-258) is used to verify the fractionation procedure (Figure 7, lower panel). It appears that HBP which is absent from native HUVECs is taken up by endothelial cells and routed to their vesicular and/or microsomal compartments in its intact form.

Colocalization of HBP and p33/gC1qR

As shown in Figure 7, HBP binds with p33/gC1qR. Because internalized HBP colocalizes with p33/gC1qR in the vesicular fraction of HUVECs double stainings for the two proteins are performed. Endothelial cells are incubated with FITC-labeled HBP for up to 24 h, fixed and double-stained for p33 using antibodies to human p33 (from rabbit) followed by a Texas red-conjugated anti-rabbit immunoglobulin (from goat). Both FITC-conjugated HBP (green) and p33/gC1qR (red) are prominent in parnuclear spots (Figure 8A). A color shift to yellow/orange in the confocal overlay and black spots in the subtraction overlay appears to indicate that at least a fraction of HBP and p33 colocalize within HUVECs (Figure 7B). Because p33/gC1qR has been demonstrated to be a mitochondrial protein, these findings seem to indicate that internalized HBP is targeted to compartments juxtaposed to or even associated with mitochondria.

Effect of hHBP on Apoptosis Induced by Removal of FCS

HUVECs are incubated for 24 hours with hHBP at the indicated concentrations. The medium is changed and the preincubation is followed by an 18 hours incubation in serum-free medium, to induce apoptosis. Control cells are incubated with M199 supplemented with 10% FCS. DNA fragmentation is measrued by a TUNEL method. The results are shown in Figure 9. They indicate that there is a decrease in apoptosis in hHBP treated cells.

Effect of hHBP on Hydrogen Peroxide Treated Cells

HUVEC cells are treated with medium (control) and medium containing hydrogen peroxide as indicated for 18 hours in the presence or absence of hHBP for 18 hours. Apoptosis is determined as above.

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as illustrations of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

Various references are cited herein, the disclosure of which are incorporated by reference in their entireties.

CLAIMS

1. A method of modulating or decreasing apoptosis in mammalian cells of a mammal selected from the group consisting of beta cells of Islets of Langerhans, endothelial cells and nerve cells, comprising administering to said mammal in need thereof, a mammalian heparin-binding protein which in glycosylated form has (i) a molecular weight of about 28 kD as determined by SDS PAGE under reducing conditions; (ii) is produced in the azurophil granules of polymorphonuclear leukocytes and (iii) is a chemoattractant for monocytes or pharmaceutically active fragment thereof in an amount effective to modulate or decrease apoptosis in said cells.

2. The method according to claim 1, in which the mammalian heparin-binding protein is a human or porcine HBP.

3. The method according to claim 1, in which the HBP has an amino acid sequence which has at least about an 80% identity with the amino acid sequence set forth in SEQ ID NO:1, 2, 5, 7, 9, or 11 or an allelic or natural variant thereof.

4. The method according to claim 1, in which the HBP is encoded by a nucleic acid sequence which hybridizes to the nucleic acid sequence set forth in SEQ ID NO:3, 4, 6, 8, 10, or 12; (ii) its complementary strand, or (iii) a subsequence of (a) or (b).

5. The method according to claim 2, in which the HBP has an amino acid sequence set forth in SEQ ID NO:1, 2, 5, 7, 9, or 11.

6. The method according to claim 1, in which the HBP is encoded by a nucleic acid sequence which hybridizes to the nucleic acid sequence set forth in SEQ ID NO:3, 4, 6, 8, 10 or 12.

7. The method according to claim 1, in which the heparin-binding protein is present in an amount of from about 10 mg to about 1 g per unit dosage form.

8. The method according to claim 1, in which the heparin-binding protein is present in an amount of about 0.1-100 mg/kg body weight.

9. The method according to claim 1, in which the heparin-binding protein is present in an amount of about 0.5-50 mg/kg body weight.

10. The method according to claim 1, in which the heparin-binding protein is present in an amount of about 1-25 mg/kg body weight.

11. A method of modulating or decreasing apoptosis in mammalian cells of a mammal selected from the group consisting of beta cells of Islets of Langerhans, endothelial cells and nerve cells, comprising administering to said mammal in need thereof a composition comprising (a) mammalian heparin binding protein which in glycosylated form has (i) a molecular weight of about 28 kD as determined by SDS PAGE under reducing conditions; (ii) is produced in the azurophil granules of polymorphonuclear leukocytes and (iii) is a chemoattractant for monocytes or pharmaceutically active fragment thereof and (b) a pharmaceutically acceptable carrier in an amount effective to modulate or decrease apoptosis in said cells.

12. A method of preventing or treating a disorder resulting from apoptosis of mammalian cells selected from the group consisting of beta cells of Islets of Langerhans, endothelial cells and nerve cells, in a mammal comprising administering to said mammal in need thereof a mammalian heparin-binding protein which in glycosylated form has (i) a molecular weight of about 28 kD as determined by SDS PAGE under reducing conditions; (ii) is produced in the azurophil granules of polymorphonuclear leukocytes and (iii) is a chemoattractant for monocytes or pharmaceutically active fragment thereof in an amount effective to modulate or decrease apoptosis in said cells.

13. The method according to claim 12, in which the disorder is selected from the group consisting of a condition of insufficient functioning of insulin production or insulin action, a

neurodegenerative disorder, a neuromuscular disorder, human immunodeficiency virus and ischemic stroke.

14. The method according to claim 1, in which the mammal is a human patient.

15. A method of preventing or treating a disorder resulting from apoptosis of mammalian cells of a mammal selected from the group consisting of beta cells of Islets of Langerhans, endothelial cells and nerve cells, comprising administering to said mammal in need thereof a composition comprising (a) mammalian heparin-binding protein which in glycosylated form ~~has (i) a molecular weight of about 28 kD as determined by SDS PAGE under reducing con-~~ditions; (ii) is produced in the azurophil granules of polymorphonuclear leukocytes and (iii) is a chemoattractant for monocytes or pharmaceutically active fragment thereof and (b) a pharmaceutically acceptable carrier in an amount effective to modulate or decrease apoptosis in said cells.

16. A composition comprising (a) mammalian heparin-binding protein which in glycosylated form has (i) a molecular weight of about 28 kD as determined by SDS PAGE under reducing conditions; (ii) is produced in the azurophil granules of polymorphonuclear leukocytes and (iii) is a chemoattractant for monocytes or pharmaceutically active fragment thereof and (b) a proteoglycan which binds to said heparin-binding protein.

17. A composition comprising (a) mammalian heparin-binding protein which in glycosylated form has (i) a molecular weight of about 28 kD as determined by SDS PAGE under reducing conditions; (ii) is produced in the azurophil granules of polymorphonuclear leukocytes and (iii) is a chemoattractant for monocytes or pharmaceutically active fragment thereof and (b) a protein which is a mammalian mitochondrial matrix targeting protein and which binds to said heparin-binding protein.

18. The composition according to claim 17 in which said mitochondrial matrix binding protein comprises a mitochondrial targeting sequence depicted in SEQ ID NO:13.

19. A method of modulating or decreasing apoptosis in mammalian cells of a mammal selected from the group consisting of beta cells of Islets of Langerhans, endothelial cells and nerve cells, comprising administering to said mammal in need thereof, the composition of claim 16 in an amount effective to modulate or decrease apoptosis in said cells.

20. A method of modulating or decreasing apoptosis in mammalian cells of a mammal selected from the group consisting of beta cells of Islets of Langerhans, endothelial cells and nerve cells, comprising administering to said mammal in need thereof the composition of claim 17 in an amount effective to modulate or decrease apoptosis in said cells.

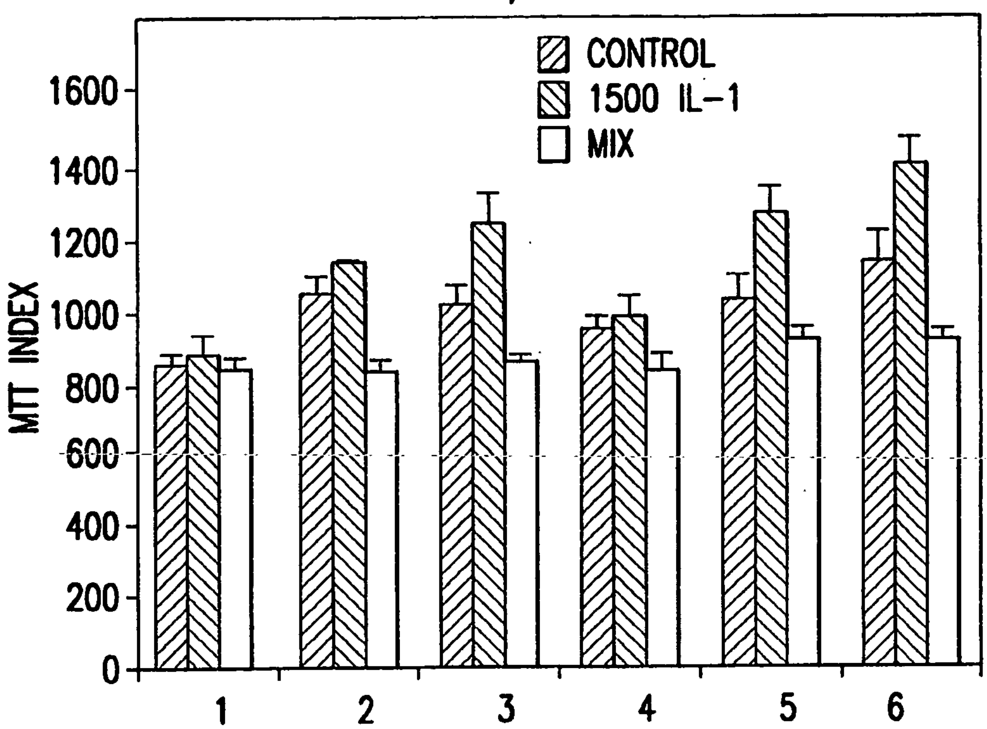

CONTROL
1500 IL-1
MIX
MTT INDEX
1600
1400
1200
1000
800
600
400
200
0
1
2
3
4
5
6

FIG.1

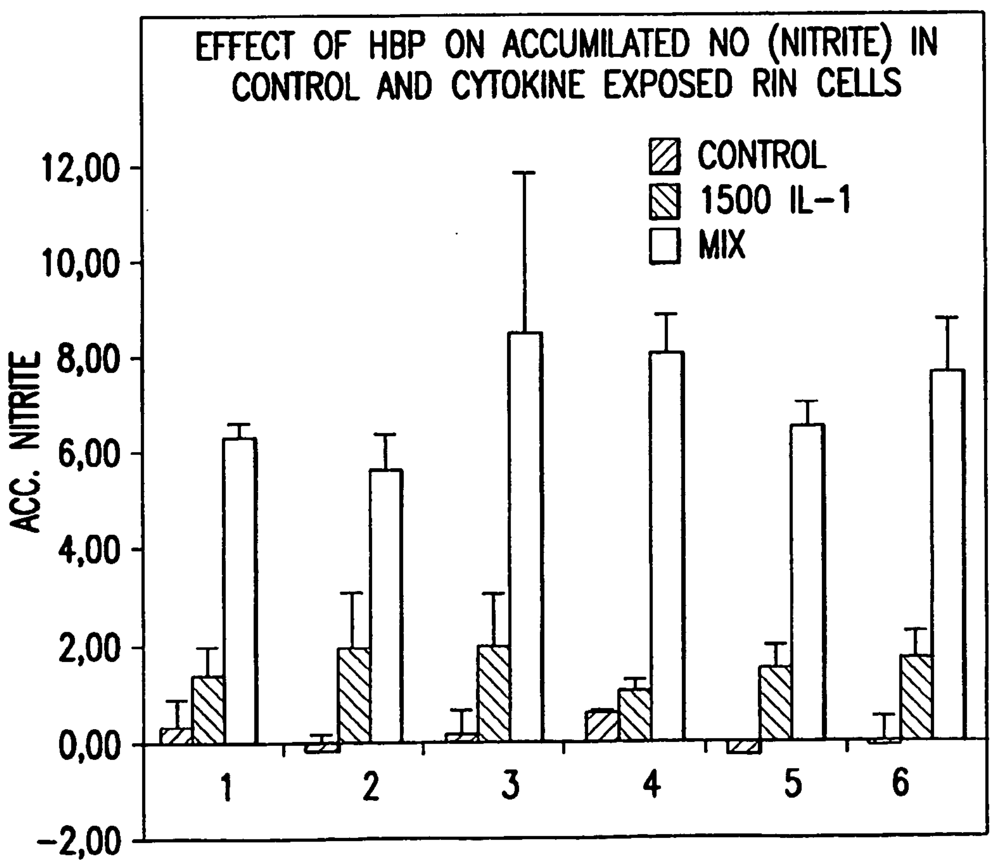

EFFECT OF HBP ON ACCUMILATED NO (NITRITE) IN CONTROL AND CYTOKINE EXPOSED RIN CELLS
CONTROL
1500 IL-1
MIX
ACC. NITRITE
12,00
10,00
8,00
6,00
4,00
2,00
0,00
-2,00
1
2
3
4
5
6

FIG.2

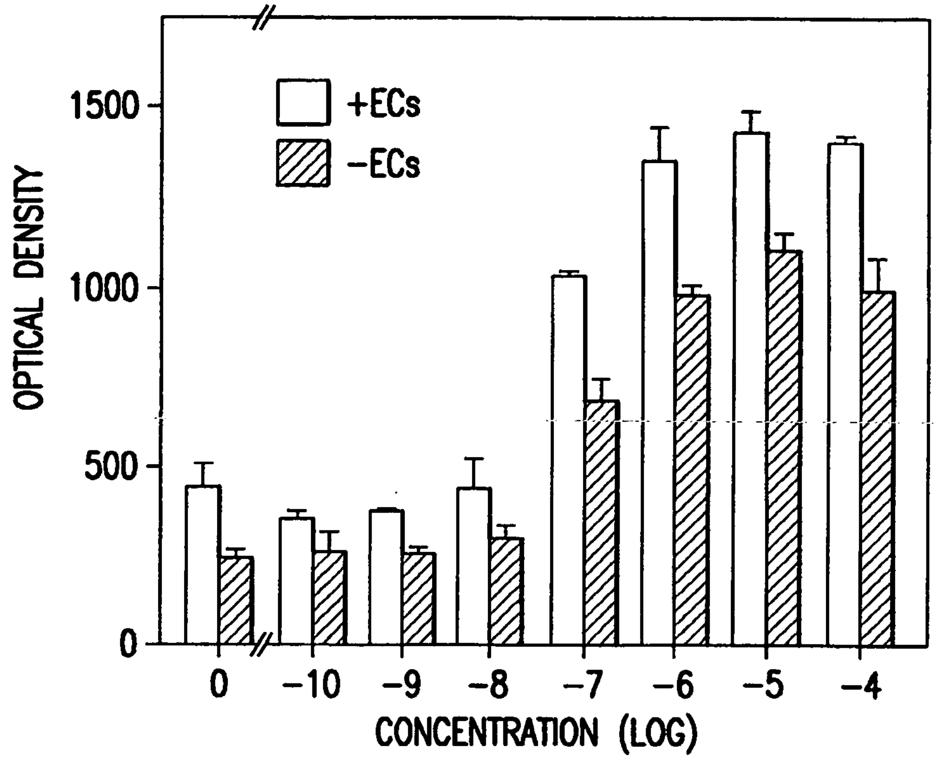

+ECs
−ECs
1500
1000
500
0
OPTICAL DENSITY
0
−10
−9
−8
−7
−6
−5
−4
CONCENTRATION (LOG)

FIG.3A

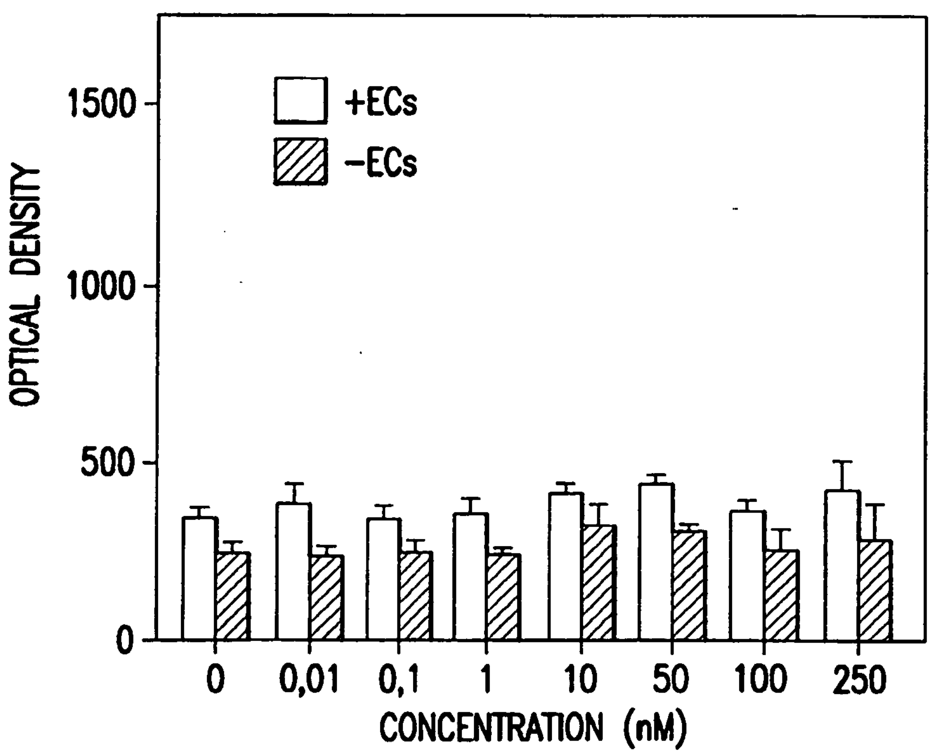

+ECs
−ECs
1500
1000
500
0
OPTICAL DENSITY
0
0,01
0,1
1
10
50
100
250
CONCENTRATION (nM)

FIG.3B

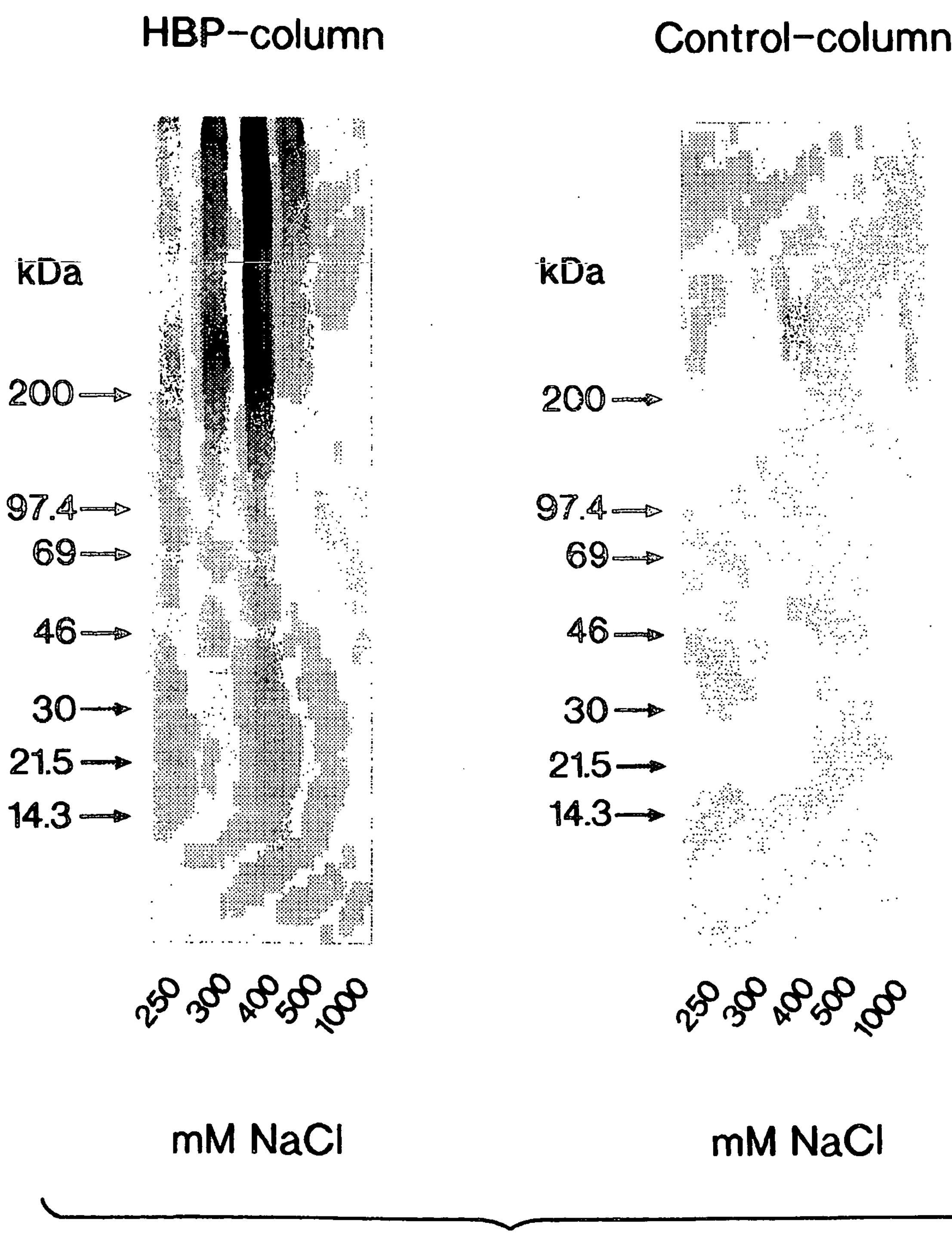

HBP-column
Control-column
kDa
200
97.4
69
46
30
21.5
14.3
kDa
200
97.4
69
46
30
21.5
14.3
250 300 400 500 1000
250 300 400 500 1000
mM NaCl
mM NaCl

FIG.4A

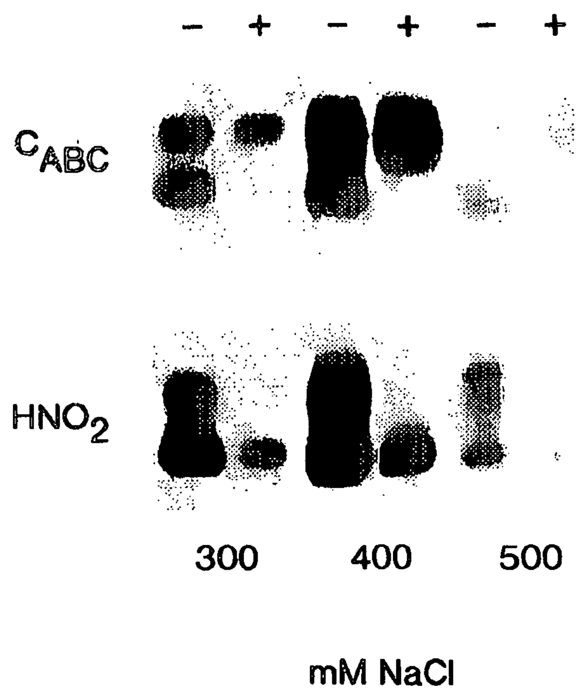


FIG.4B

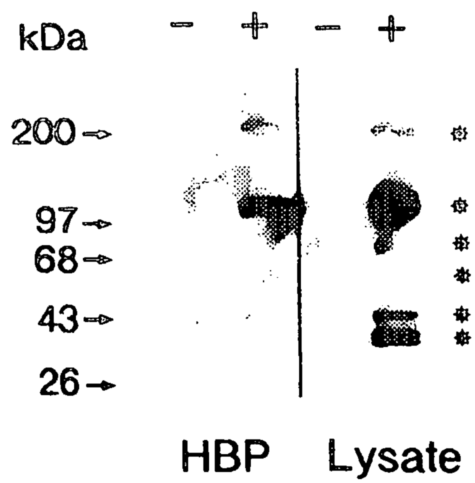


FIG.4C

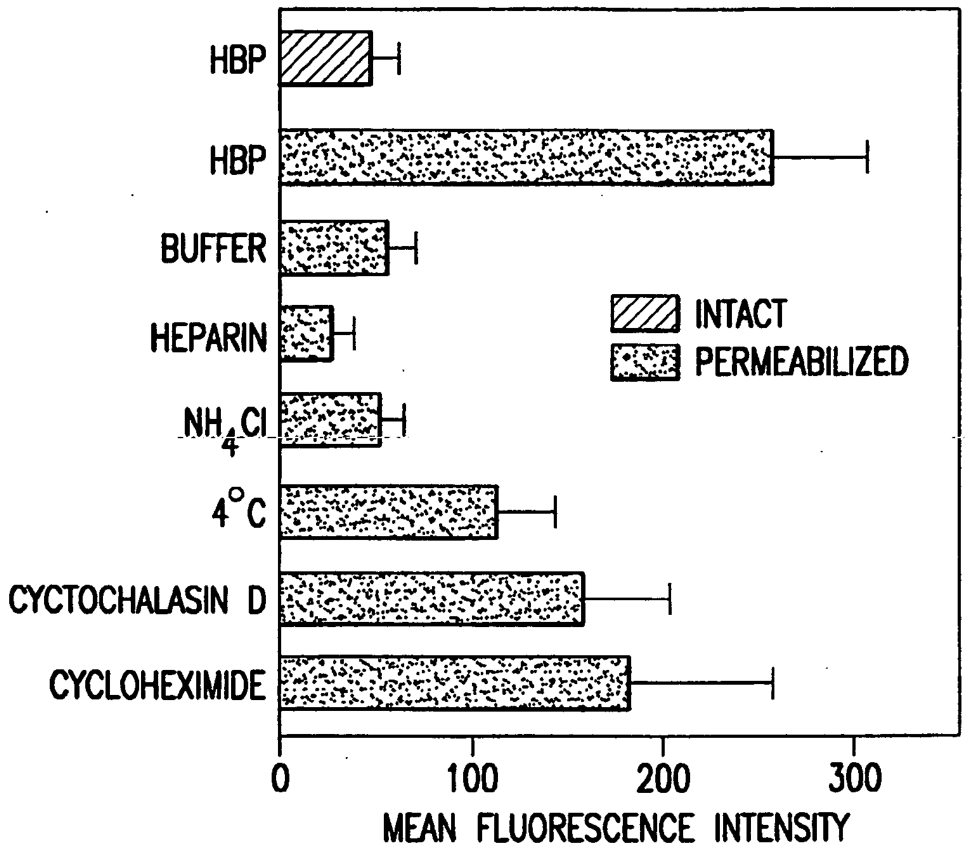

HBP
HBP
BUFFER
HEPARIN
NH_4Cl
4°C
CYCTOCHALASIN D
CYCLOHEXIMIDE
INTACT
PERMEABILIZED
0
100
200
300
MEAN FLUORESCENCE INTENSITY

FIG. 5A

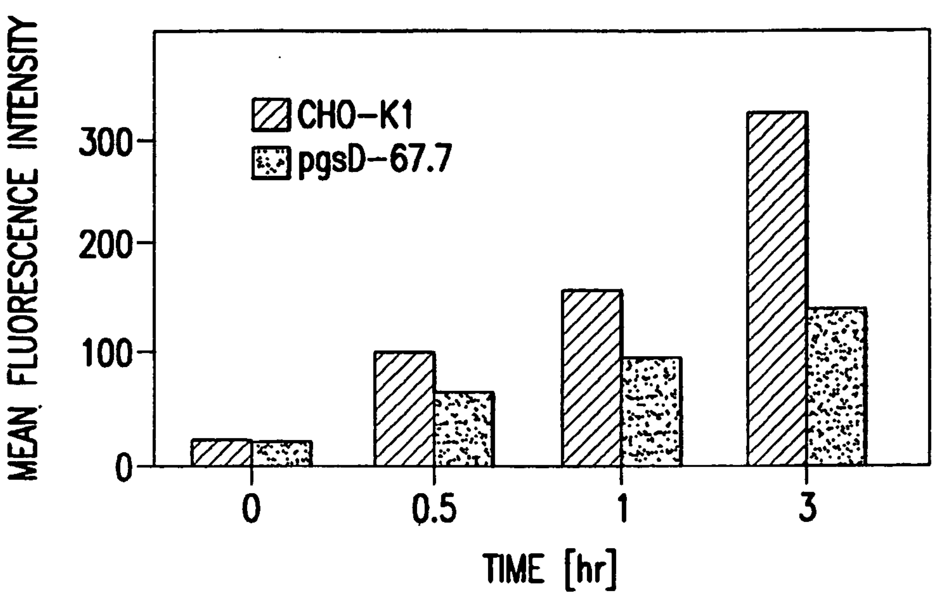

MEAN FLUORESCENCE INTENSITY
CHO-K1
pgsD-67.7
0
100
200
300
0
0.5
1
3
TIME [hr]

FIG. 5B

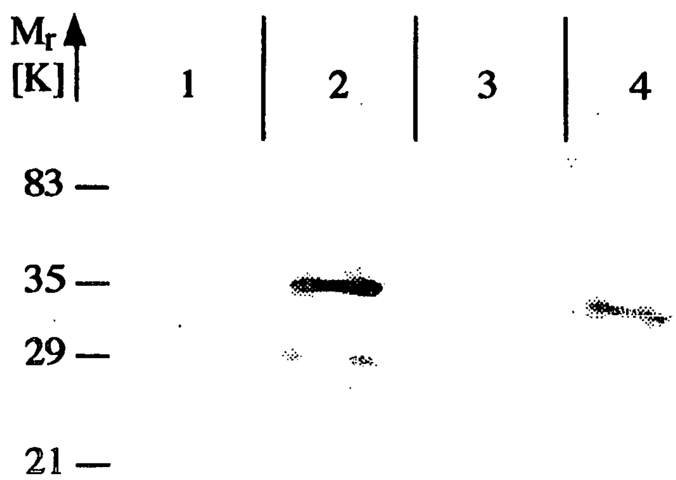


FIG.6A

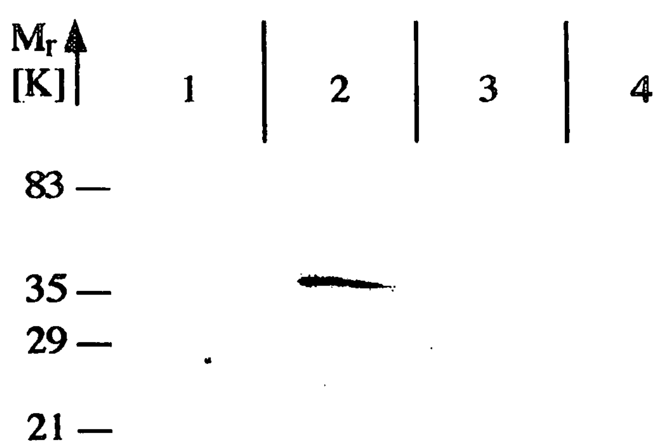


FIG.6B

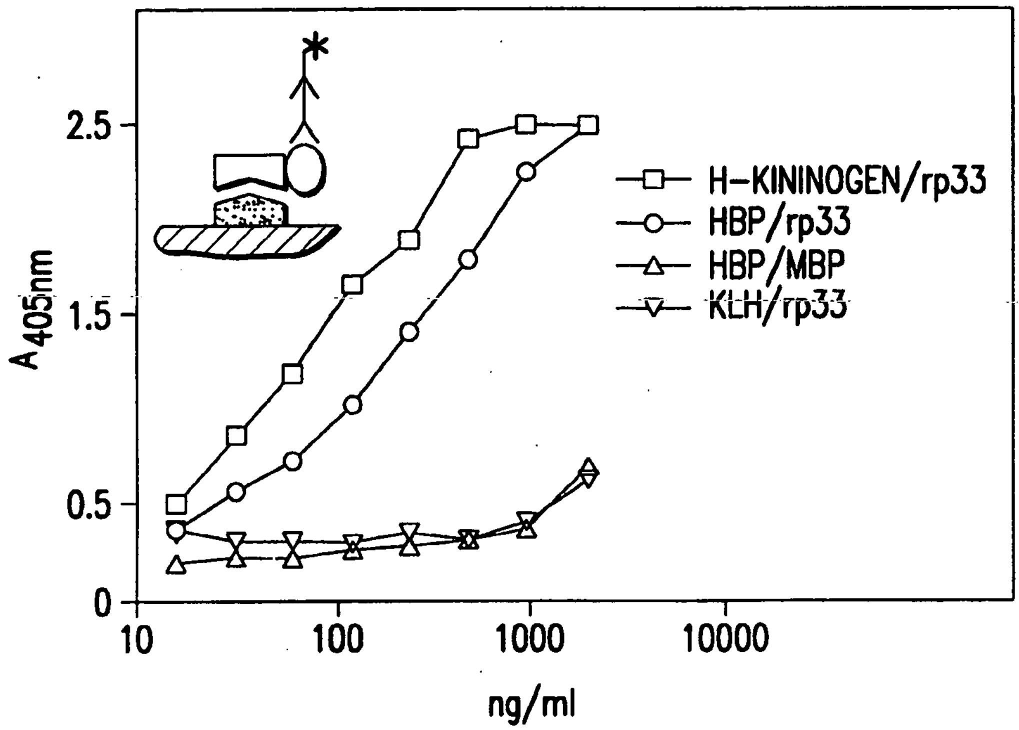
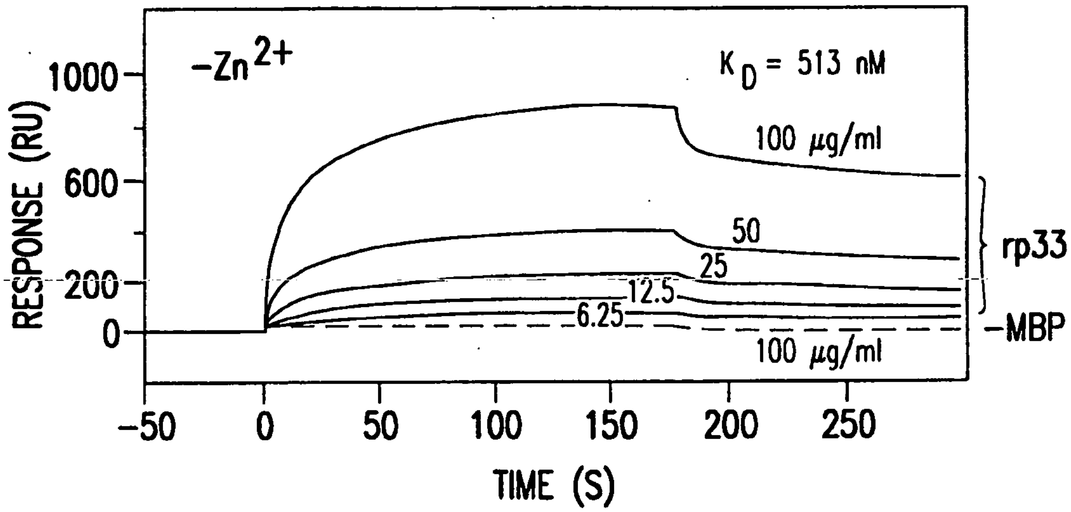
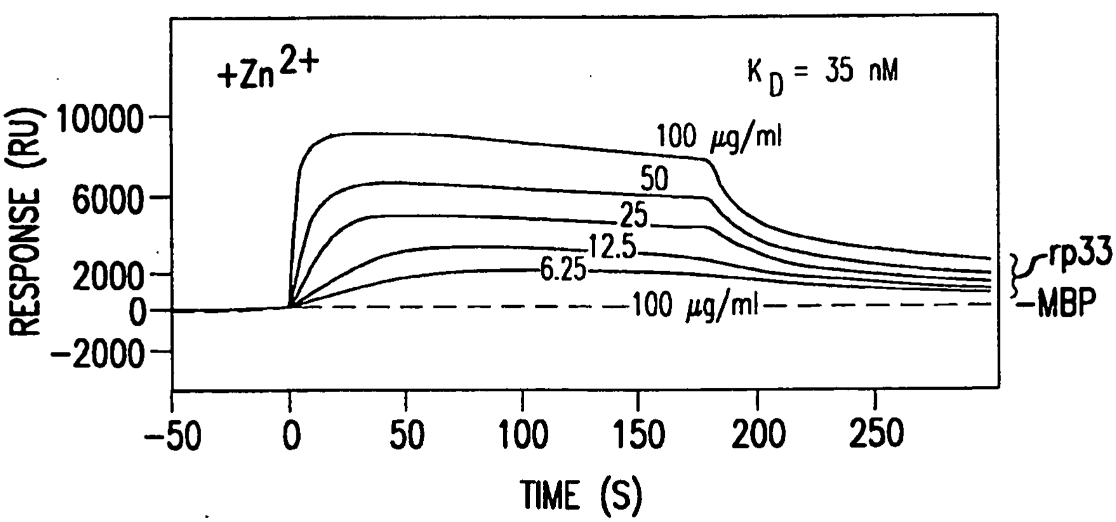

2.5
1.5
0.5
0
10
100
1000
10000
A_{405nm}
ng/ml
H-KININOGEN/rp33
HBP/rp33
HBP/MBP
KLH/rp33

FIG. 7A



$-Zn^{2+}$
K_D = 513 nM
100 μg/ml
50
25
12.5
6.25
100 μg/ml
rp33
MBP
RESPONSE (RU)
1000
600
200
0
-50
0
50
100
150
200
250
TIME (S)

FIG. 7B



$+Zn^{2+}$
K_D = 35 nM
100 μg/ml
50
25
12.5
6.25
100 μg/ml
rp33
MBP
RESPONSE (RU)
10000
6000
2000
0
-2000
-50
0
50
100
150
200
250
TIME (S)

FIG.7C

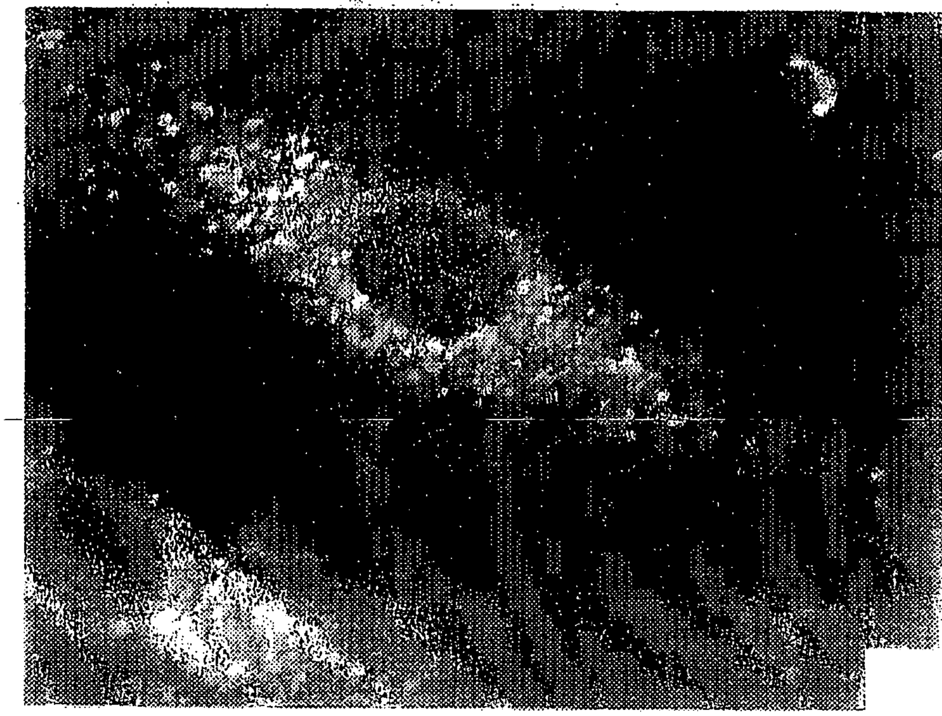


FIG.8B

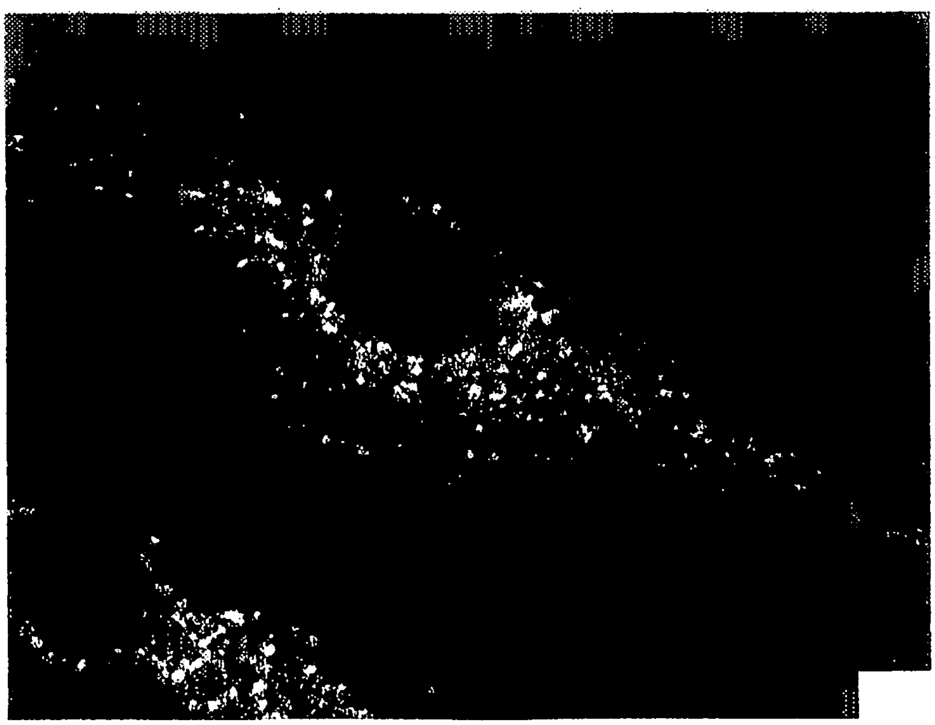


FIG.8A

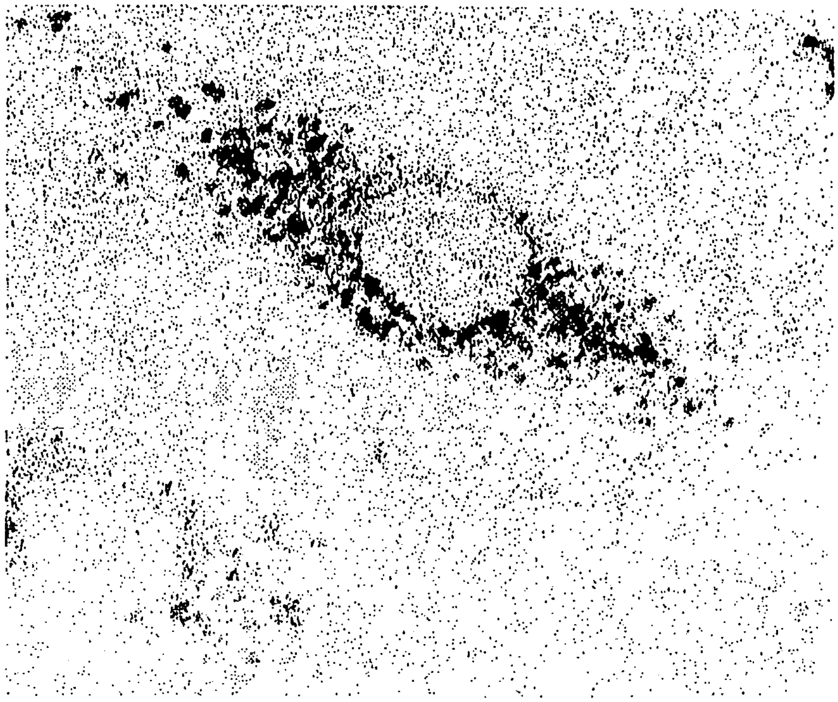


FIG.8D

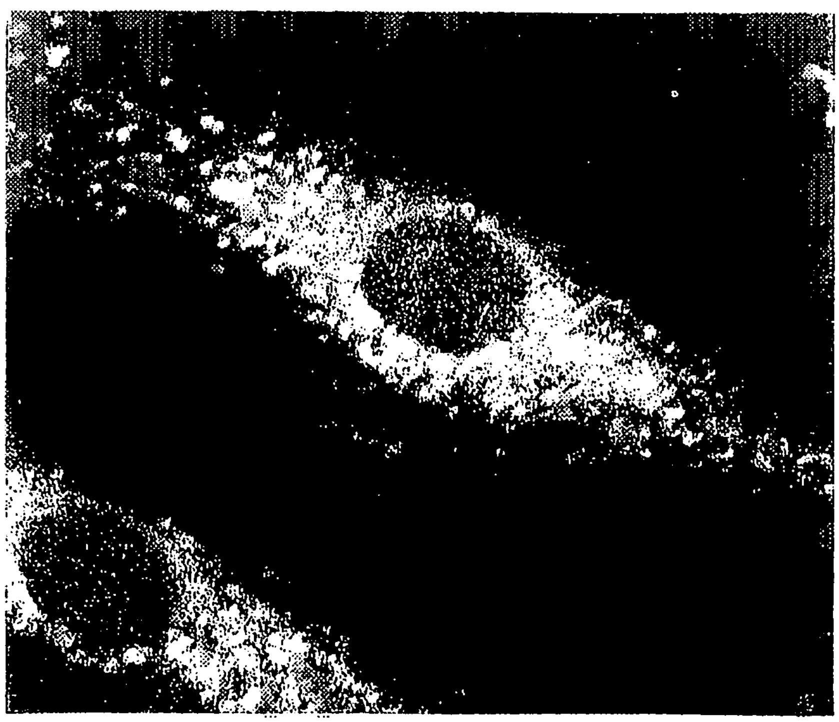


FIG.8C

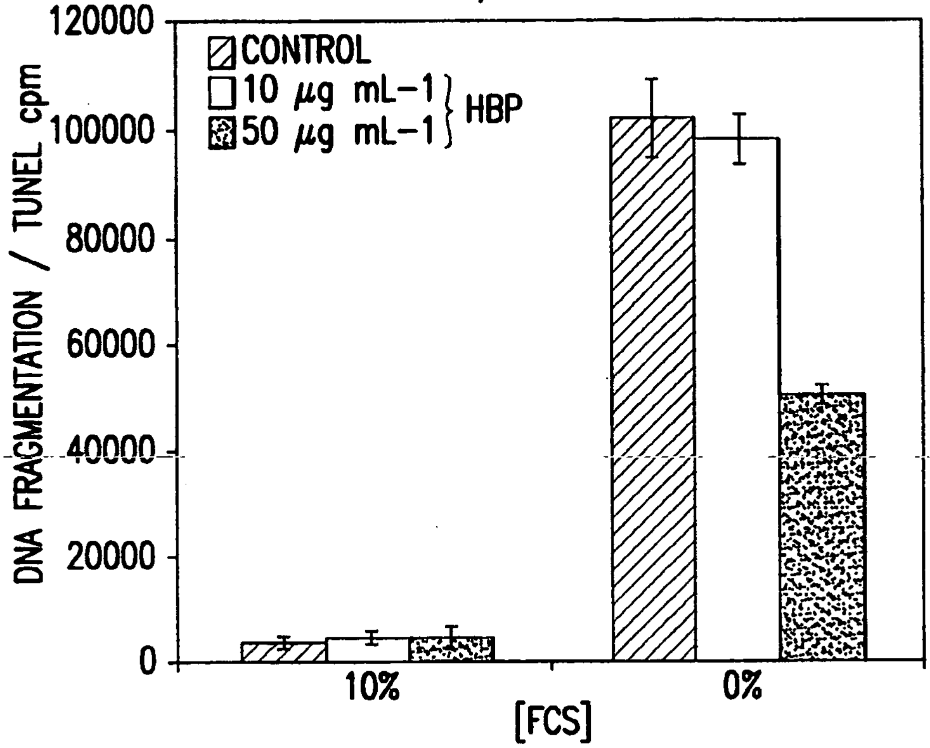

120000
100000
80000
60000
40000
20000
0
DNA FRAGMENTATION / TUNEL cpm
CONTROL
10 μg mL−1
50 μg mL−1
HBP
10%
0%
[FCS]

FIG.9

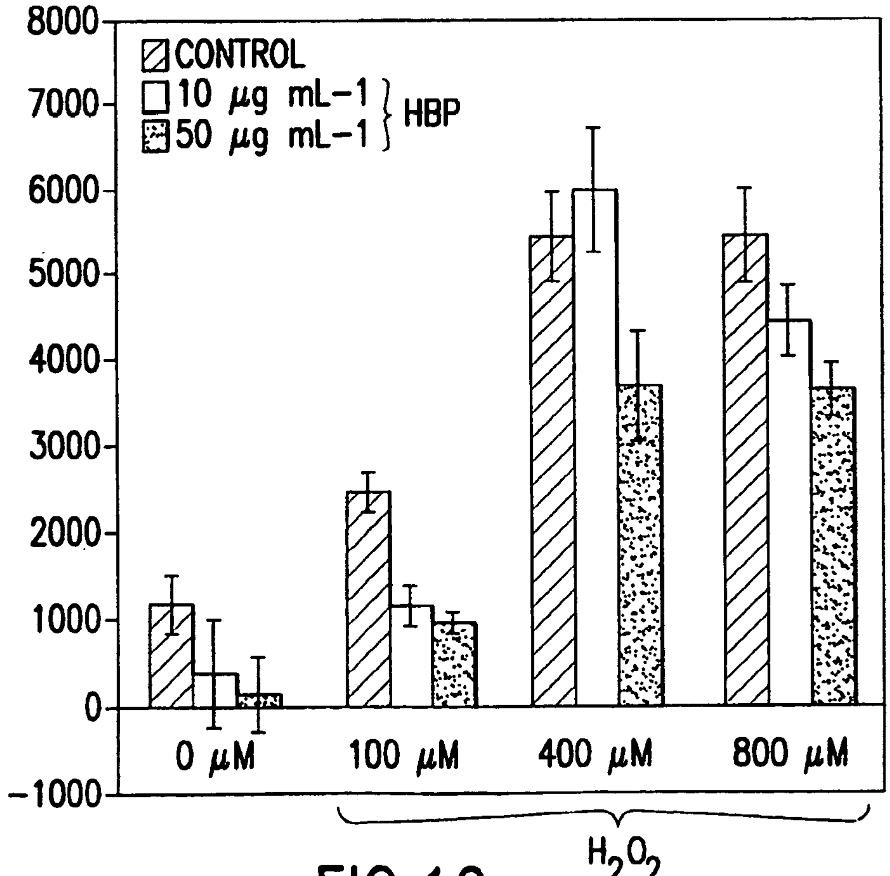

8000
7000
6000
5000
4000
3000
2000
1000
0
−1000
CONTROL
10 μg mL−1
50 μg mL−1
HBP
0 μM
100 μM
400 μM
800 μM
H_2O_2

FIG.10

SEQUENCE LISTING

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<120> Use of Heparin-Binding Protein For The
Modulation or Prophylaxis of Apoptosis of Mammalian Cells

<130> 5426-WO,JWKi

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<212> PRT
<213> Homo sapiens

<400> 1

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            20                  25                  30
Pro His Glu Pro Arg Pro His Glu Leu Glu Ala Leu Ala Ser Glu Arg
        35                  40                  45
Ile Leu Glu Gly Leu Asn Ala Ser Asn Gly Leu Asn Gly Leu Tyr Ala
    50                  55                  60
Arg Gly His Ile Ser Pro His Glu Cys Tyr Ser Gly Leu Tyr Gly Leu
65                  70                  75                  80
Tyr Ala Leu Ala Leu Glu Ile Leu Glu His Ile Ser Ala Leu Ala Ala
                85                  90                  95
Arg Gly Pro His Glu Val Ala Leu Met Glu Thr Thr His Arg Ala Leu
            100                 105                 110
Ala Ala Leu Ala Ser Glu Arg Cys Tyr Ser Pro His Glu Gly Leu Asn
        115                 120                 125
Ser Glu Arg Gly Leu Asn Ala Ser Asn Pro Arg Gly Leu Tyr Val Ala
    130                 135                 140
Leu Ser Glu Arg Thr His Arg Val Ala Leu Val Ala Leu Leu Glu Gly
145                 150                 155                 160
Leu Tyr Ala Leu Ala Thr Tyr Arg Ala Ser Pro Leu Glu Ala Arg Gly
                165                 170                 175
Ala Arg Gly Ala Arg Gly Gly Leu Ala Arg Gly Gly Leu Asn Ser Glu
            180                 185                 190
Arg Ala Arg Gly Gly Leu Asn Thr His Arg Pro His Glu Ser Glu Arg
        195                 200                 205
Ile Leu Glu Ser Glu Arg Ser Glu Arg Met Glu Thr Ser Glu Arg Gly
    210                 215                 220
Leu Ala Ser Asn Gly Leu Tyr Thr Tyr Arg Ala Ser Pro Pro Arg Gly
225                 230                 235                 240
Leu Asn Gly Leu Asn Ala Ser Asn Leu Glu Ala Ser Asn Ala Ser Pro
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                245                 250                 255
Leu Glu Met Glu Thr Leu Glu Leu Glu Gly Leu Asn Leu Glu Ala Ser
            260                 265                 270
Pro Ala Arg Gly Gly Leu Ala Leu Ala Ala Ser Asn Leu Glu Thr His
        275                 280                 285
Arg Ser Glu Arg Ser Glu Arg Val Ala Leu Thr His Arg Ile Leu Glu
    290                 295                 300
Leu Glu Pro Arg Leu Glu Pro Arg Leu Glu Gly Leu Asn Ala Ser Asn
305                 310                 315                 320
Ala Leu Ala Thr His Arg Val Ala Leu Gly Leu Ala Leu Ala Gly Leu
                325                 330                 335
Tyr Thr His Arg Ala Arg Gly Cys Tyr Ser Gly Leu Asn Val Ala Leu
            340                 345                 350
Ala Leu Ala Gly Leu Tyr Thr Arg Pro Gly Leu Tyr Ser Glu Arg Gly
        355                 360                 365
Leu Asn Ala Arg Gly Ser Glu Arg Gly Leu Tyr Gly Leu Tyr Ala Arg
    370                 375                 380
Gly Leu Glu Ser Glu Arg Ala Arg Gly Pro His Glu Pro Arg Ala Arg
385                 390                 395                 400
Gly Pro His Glu Val Ala Leu Ala Ser Asn Val Ala Leu Thr His Arg
                405                 410                 415
Val Ala Leu Thr His Arg Pro Arg Gly Leu Ala Ser Pro Gly Leu Asn
            420                 425                 430
Cys Tyr Ser Ala Arg Gly Pro Arg Ala Ser Asn Ala Ser Asn Val Ala
        435                 440                 445
Leu Cys Tyr Ser Thr His Arg Gly Leu Tyr Val Ala Leu Leu Glu Thr
    450                 455                 460
His Arg Ala Arg Gly Ala Arg Gly Gly Leu Tyr Gly Leu Tyr Ile Leu
465                 470                 475                 480
Glu Cys Tyr Ser Ala Ser Asn Gly Leu Tyr Ala Ser Pro Gly Leu Tyr
                485                 490                 495
Gly Leu Tyr Thr His Arg Pro Arg Leu Glu Val Ala Leu Cys Tyr Ser
            500                 505                 510
Gly Leu Gly Leu Tyr Leu Glu Ala Leu Ala His Ile Ser Gly Leu Tyr
        515                 520                 525
Val Ala Leu Ala Leu Ala Ser Glu Arg Pro His Glu Ser Glu Arg Leu
    530                 535                 540
Glu Gly Leu Tyr Pro Arg Cys Tyr Ser Gly Leu Tyr Ala Arg Gly Gly
545                 550                 555                 560
Leu Tyr Pro Arg Ala Ser Pro Pro His Glu Pro His Glu Thr His Arg
                565                 570                 575
Ala Arg Gly Val Ala Leu Ala Leu Ala Leu Glu Pro His Glu Ala Arg
            580                 585                 590
Gly Ala Ser Pro Thr Arg Pro Ile Leu Glu Ala Ser Pro Gly Leu Tyr
        595                 600                 605
Val Ala Leu Leu Glu Ala Ser Asn Ala Ser Asn Pro Arg Gly Leu Tyr
    610                 615                 620
Pro Arg Gly Leu Tyr Pro Arg Ala Leu Ala
625                 630
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<210> 2
<211> 621
<212> PRT
<213> Homo sapiens

<400> 2

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 1               5                  10                  15
```

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        20                  25                  30
His Glu Pro Arg Pro His Glu Leu Glu Ala Leu Ala Ser Glu Arg Ile
    35                  40                  45
Leu Glu Gly Leu Asn Leu Tyr Ser Gly Leu Asn Gly Leu Tyr Ala Arg
    50                  55                  60
Gly Pro Arg Pro His Glu Cys Tyr Ser Ala Leu Ala Gly Leu Tyr Ala
65                  70                  75                  80
Leu Ala Leu Glu Val Ala Leu His Ile Ser Pro Arg Ala Arg Gly Pro
                85                  90                  95
His Glu Val Ala Leu Leu Glu Thr His Arg Ala Leu Ala Ala Leu Ala
            100                 105                 110
Ser Glu Arg Cys Tyr Ser Pro His Glu Ala Arg Gly Gly Leu Tyr Leu
        115                 120                 125
Tyr Ser Ala Ser Asn Ser Glu Arg Gly Leu Tyr Ser Glu Arg Ala Leu
    130                 135                 140
Ala Ser Glu Arg Val Ala Leu Val Ala Leu Leu Glu Gly Leu Tyr Ala
145                 150                 155                 160
Leu Ala Thr Tyr Arg Ala Ser Pro Leu Glu Ala Arg Gly Gly Leu Asn
                165                 170                 175
Gly Leu Asn Gly Leu Gly Leu Asn Ser Glu Arg Ala Arg Gly Gly Leu
            180                 185                 190
Asn Thr His Arg Pro His Glu Ser Glu Arg Ile Leu Glu Ala Arg Gly
        195                 200                 205
Ser Glu Arg Ile Leu Glu Ser Glu Arg Gly Leu Asn Ala Ser Asn Gly
    210                 215                 220
Leu Tyr Thr Tyr Arg Ala Ser Pro Pro Arg Ala Arg Gly Gly Leu Asn
225                 230                 235                 240
Ala Ser Asn Leu Glu Ala Ser Asn Ala Ser Pro Val Ala Leu Leu Glu
                245                 250                 255
Leu Glu Leu Glu Gly Leu Asn Leu Glu Ala Ser Pro Ala Arg Gly Gly
            260                 265                 270
Leu Ala Leu Ala Ala Arg Gly Leu Glu Thr His Arg Pro Arg Ser Glu
        275                 280                 285
Arg Val Ala Leu Ala Leu Ala Leu Glu Val Ala Leu Pro Arg Leu Glu
    290                 295                 300
Pro Arg Pro Arg Gly Leu Asn Ala Ser Asn Ala Leu Ala Thr His Arg
305                 310                 315                 320
Val Ala Leu Gly Leu Ala Leu Ala Gly Leu Tyr Thr His Arg Ala Ser
                325                 330                 335
Asn Cys Tyr Ser Gly Leu Asn Val Ala Leu Gly Leu Ala Leu Ala Gly
            340                 345                 350
Leu Tyr Thr Arg Pro Gly Leu Tyr Thr His Arg Gly Leu Asn Ala Arg
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Gly Leu Glu Ala Arg Gly Ala Arg Gly Leu Glu Pro His Glu Ser Glu
    370                 375                 380
Arg Ala Arg Gly Pro His Glu Pro Arg Ala Arg Gly Val Ala Leu Leu
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Glu Ala Ser Asn Val Ala Leu Thr His Arg Val Ala Leu Thr His Arg
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Ser Glu Arg Ala Ser Asn Pro Arg Cys Tyr Ser Leu Glu Pro Arg Ala
            420                 425                 430
Arg Gly Ala Ser Pro Met Glu Thr Cys Tyr Ser Ile Leu Glu Gly Leu
        435                 440                 445
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Gly Leu Tyr Ala Arg Gly Ile Leu Glu Ser Glu Arg Gly Leu Asn Gly
465                 470                 475                 480
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545 550 555 560
Leu Tyr Pro His Glu Pro His Glu Thr His Arg Ala Arg Gly Val Ala
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ctcaccagca gcgtgacgat actgccactg cctctgcaga acgccacggt ggaagccggc 360
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cccaggttcg tcaacgtgac tgtgaccccc gaggaccagt gtcgccccaa caacgtgtgc 480
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cgcttcgtga tgaccgcggc cagctgcttc caaagccaga accccggggt tagcaccgtg    180
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agcagcatga gcgagaatgg ctacgacccc cagcagaacc tgaacgacct gatgctgctt    300
cagctggacc gtgaggccaa cctcaccagc agcgtgacga tactgccact gcctctgcag    360
aacgccacgg tggaagccgg caccagatgc caggtggccg gctgggggag ccagcgcagt    420
ggggggcgtc tctcccgttt tcccaggttc gtcaacgtga ctgtgacccc cgaggaccag    480
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gacgggggca ccccccctcgt ctgcgagggc ctggcccacg gcgtggcctc cttttccctg    600
gggccctgtg gccgaggccc tgacttcttc acccgagtgg cgctcttccg agactggatc    660
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<211> 232
<212> PRT
<213> Homo sapiens

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            20                  25                  30
Cys Gly Gly Ala Leu Ile His Ala Arg Phe Val Met Thr Ala Ala Ser
        35                  40                  45
Cys Phe Gln Ser Gln Asn Pro Gly Val Ser Thr Val Val Leu Gly Ala
    50                  55                  60
Tyr Asp Leu Arg Arg Arg Glu Arg Gln Ser Arg Gln Thr Phe Ser Ile
65                  70                  75                  80
Ser Ser Met Ser Glu Asn Gly Tyr Asp Pro Gln Gln Asn Leu Asn Asp
                85                  90                  95
Leu Met Leu Leu Gln Leu Asp Arg Glu Ala Asn Leu Thr Ser Ser Val
            100                 105                 110
Thr Ile Leu Pro Leu Pro Leu Gln Asn Ala Thr Val Glu Ala Gly Thr
        115                 120                 125
Arg Cys Gln Val Ala Gly Trp Gly Ser Gln Arg Ser Gly Gly Arg Leu
    130                 135                 140
Ser Arg Phe Pro Arg Phe Val Asn Val Thr Val Thr Pro Glu Asp Gln
145                 150                 155                 160
Cys Arg Pro Asn Asn Val Cys Thr Gly Val Leu Thr Arg Arg Gly Gly
                165                 170                 175
Ile Cys Asn Gly Asp Gly Gly Thr Pro Leu Val Cys Glu Gly Leu Ala
            180                 185                 190
His Gly Val Ala Ser Phe Ser Leu Gly Pro Cys Gly Arg Gly Pro Asp
        195                 200                 205
Phe Phe Thr Arg Val Ala Leu Phe Arg Asp Trp Ile Asp Gly Val Leu
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<213> homo sapiens

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agcatgagcg agaatggcta cgacccccag cagaacctga acgacctgat gctgcttcag     360
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gggggcaccc ccctcgtctg cgagggcctg gcccacggcg tggcctcctt ttccctgggg     660
ccctgtggcc gaggccctga cttcttcacc cgagtggcgc tcttccgaga ctggatcgat     720
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<212> PRT
<213> Homo sapiens

<400> 8

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            20                  25                  30
Ala Arg Phe Val Met Thr Ala Ala Ser Cys Phe Gln Ser Gln Asn Pro
        35                  40                  45
Gly Val Ser Thr Val Val Leu Gly Ala Tyr Asp Leu Arg Arg Arg Glu
    50                  55                  60
Arg Gln Ser Arg Gln Thr Phe Ser Ile Ser Ser Met Ser Glu Asn Gly
65                  70                  75                  80
Tyr Asp Pro Gln Gln Asn Leu Asn Asp Leu Met Leu Leu Gln Leu Asp
                85                  90                  95
Arg Glu Ala Asn Leu Thr Ser Ser Val Thr Ile Leu Pro Leu Pro Leu
            100                 105                 110
Gln Asn Ala Thr Val Glu Ala Gly Thr Arg Cys Gln Val Ala Gly Trp
        115                 120                 125
Gly Ser Gln Arg Ser Gly Gly Arg Leu Ser Arg Phe Pro Arg Phe Val
    130                 135                 140
Asn Val Thr Val Thr Pro Glu Asp Gln Cys Arg Pro Asn Asn Val Cys
145                 150                 155                 160
Thr Gly Val Leu Thr Arg Arg Gly Gly Ile Cys Asn Gly Asp Gly Gly
                165                 170                 175
Thr Pro Leu Val Cys Glu Gly Leu Ala His Gly Val Ala Ser Phe Ser
            180                 185                 190
Leu Gly Pro Cys Gly Arg Gly Pro Asp Phe Phe Thr Arg Val Ala Leu
        195                 200                 205
Phe Arg Asp Trp Ile Asp Gly Val Leu Asn Asn Pro Gly Pro Gly Pro
    210                 215                 220
Ala
225
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    <212> PRT
    <213> Sus scrofa

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Phe Leu Ala Ser Ile Gln Lys Gln Gly Arg Pro Phe Cys Ala Gly Ala
        35                  40                  45
Leu Val His Pro Arg Phe Val Leu Thr Ala Ala Ser Cys Phe Arg Gly
    50                  55                  60
Lys Asn Ser Gly Ser Ala Ser Val Val Leu Gly Ala Tyr Asp Leu Arg
65                  70                  75                  80
Gln Gln Glu Gln Ser Arg Gln Thr Phe Ser Ile Arg Ser Ile Ser Gln
                85                  90                  95
Asn Gly Tyr Asp Pro Arg Gln Asn Leu Asn Asp Val Leu Leu Leu Gln
            100                 105                 110
Leu Asp Arg Glu Ala Arg Leu Thr Pro Ser Val Ala Leu Val Pro Leu
        115                 120                 125
Pro Pro Gln Asn Ala Thr Val Glu Ala Gly Thr Asn Cys Gln Val Ala
    130                 135                 140
Gly Trp Gly Thr Gln Arg Leu Arg Arg Leu Phe Ser Arg Phe Pro Arg
145                 150                 155                 160
Val Leu Asn Val Thr Val Thr Ser Asn Pro Cys Leu Pro Arg Asp Met
                165                 170                 175
Cys Ile Gly Val Phe Ser Arg Arg Gly Arg Ile Ser Gln Gly Asp Arg
            180                 185                 190
Gly Thr Pro Leu Val Cys Asn Gly Leu Ala Gln Gly Val Ala Ser Phe
        195                 200                 205
Leu Arg Arg Arg Phe Arg Arg Ser Ser Gly Phe Phe Thr Arg Val Ala
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Leu Phe Arg Asn Trp Ile Asp Ser Val Leu Asn Asn Pro Pro Ala
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<400> 12

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Ala Gln Pro Gln Glu Phe Pro Phe Leu Ala Ser Ile Gln Lys Gln Gly
        35                  40                  45
Arg Pro Phe Cys Ala Gly Ala Leu Val His Pro Arg Phe Val Leu Thr
    50                  55                  60
Ala Ala Ser Cys Phe Arg Gly Lys Asn Ser Gly Ser Ala Ser Val Val
65                  70                  75                  80
Leu Gly Ala Tyr Asp Leu Arg Gln Gln Glu Gln Ser Arg Gln Thr Phe
                85                  90                  95
Ser Ile Arg Ser Ile Ser Gln Asn Gly Tyr Asp Pro Arg Gln Asn Leu
            100                 105                 110
Asn Asp Val Leu Leu Leu Gln Leu Asp Arg Glu Ala Arg Leu Thr Pro
        115                 120                 125
Ser Val Ala Leu Val Pro Leu Pro Pro Gln Asn Ala Thr Val Glu Ala
    130                 135                 140
Gly Thr Asn Cys Gln Val Ala Gly Trp Gly Thr Gln Arg Leu Arg Arg
145                 150                 155                 160
Leu Phe Ser Arg Phe Pro Arg Val Leu Asn Val Thr Val Thr Ser Asn
                165                 170                 175
Pro Cys Leu Pro Arg Asp Met Cys Ile Gly Val Phe Ser Arg Arg Gly
            180                 185                 190
Arg Ile Ser Gln Gly Asp Arg Gly Thr Pro Leu Val Cys Asn Gly Leu
        195                 200                 205
Ala Gln Gly Val Ala Ser Phe Leu Arg Arg Arg Phe Arg Arg Ser Ser
    210                 215                 220
Gly Phe Phe Thr Arg Val Ala Leu Phe Arg Asn Trp Ile Asp Ser Val
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<210> 14
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<220>
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<400> 14
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<210> 15
<211> 32
<212> PRT
<213> Homo sapiens

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<211> 281
<212> PRT
<213> homo sapiens

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Gln Pro Ala Pro Arg Leu Cys Thr Arg Pro Phe Gly Leu Leu Ser Val
35 40 45
Arg Ala Gly Ser Glu Arg Arg Pro Gly Leu Leu Arg Pro Arg Gly Pro
50 55 60
Cys Ala Cys Gly Cys Gly Cys Gly Ser Leu His Thr Asp Gly Asp Lys
65 70 75 80
Ala Phe Val Asp Phe Leu Ser Asp Glu Ile Lys Glu Glu Arg Lys Ile
85 90 95
Gln Lys His Lys Thr Leu Pro Lys Met Ser Gly Gly Trp Glu Leu Glu
100 105 110
Leu Asn Gly Thr Glu Ala Lys Leu Val Arg Lys Val Ala Gly Glu Lys
115 120 125
Ile Thr Val Thr Phe Asn Ile Asn Asn Ser Ile Pro Pro Thr Phe Asp
130 135 140
Gly Glu Glu Glu Pro Ser Gln Gly Gln Lys Val Glu Glu Gln Glu Pro
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Glu Leu Thr Ser Thr Pro Asn Phe Val Val Glu Val Ile Lys Asn Asp
165 170 175
Asp Gly Lys Lys Ala Leu Val Leu Asp Cys His Tyr Pro Glu Asp Glu

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            180                 185                 190
Val Gly Gln Glu Asp Glu Ala Glu Ser Asp Ile Phe Ser Ile Arg Glu
        195                 200                 205
Ser Phe Gln Ser Thr Gly Glu Ser Glu Trp Lys Asp Thr Asn Tyr Thr
    210                 215                 220
Leu Asn Thr Asp Ser Leu Asp Trp Ala Leu Tyr Asp His Leu Met Asp
225                 230                 235                 240
Phe Leu Ala Asp Arg Gly Val Asp Asn Thr Phe Ala Asp Glu Leu Val
                245                 250                 255
Glu Leu Ser Thr Ala Leu Glu His Gln Glu Tyr Ile Thr Phe Leu Glu
            260                 265                 270
Asp Leu Lys Ser Phe Val Lys Ser Gln
        275                 280
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 98/00510

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A61K 38/18, C07K 14/47
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9628544 A1 (NOVO NORDISK A/S), 19 Sept 1996 (19.09.96), page 2, line 20 - line 23; page 7, line 6 - line 10; page 8, line 5 - line 16 --	1-20
X	Dialog Information Services, File 73, EMBASE, Dialog accession no. 05701574, EMBASE accession no. 1994116083, Small D.H. et al: "A heparin-binding domain in the amyloid protein precursor of Alzheimer's disease is involved in the regulation of neurite outgrowth", Journal of Neuroscience (J. NEUROSCI.) (United States) 1994, 14/4 (2117-2127) --	1-20

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 February 1999	08 -03- 1999
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Palmcrantz Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 98/00510

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, File 155, Dialog accession no. 09343439, MEDLINE, Medline accession no. 98042252, Barasch J et al: "Ureteric bud cells secrete multiple factors, including bFGF, which resuce renal progenitors from apoptosis", Am J Physiol (UNITED STATES) Nov 1997, 273 (5 Pt 2) pF757-67 --	1-20
P,X	Dialog Information Services, File 155, MEDLINE, Dialog accession no. 09354776, Medline accession no. 98049575, Takemura T. et al: "The membrane-bound form heparin-binding epidermal growth factor-like growth factor promotes survival of cultures renal epithelialcells", J Biol Chem (UNITED STATES) Dec 5 1997, 272 (49) p 31036-42 --	1-20
P,X	Dialog Information Services, File 155, MEDLINE, Dialog accession no.09354396, Medline accession no. 98060597, Zushi S et al: "Role of heparin-binding EGF-related peptides in proliferation and apoptosis of activated ras-stimulated intestinal epithelial cells", Int J Cancer (UNITED STATES) Dec 10 1997, 73 (6) p 917-23 --	1-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK98/00510

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [x] Claims Nos.: 1-15,19-20
because they relate to subject matter not required to be searched by this Authority, namely:

Claims 1-15 and 19-20 relate to methods of treatment of the human or animal body by therapy Rule.39.1.(iv). Nevertheless, a search has been executed for these claims. The search has been based on the alleged effects of the compounds/compositions.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

02/02/99

International application No.
PCT/DK 98/00510

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9628544 A1	19/09/96	AU 4784896 A	02/10/96
		CA 2214799 A	19/09/96
		CN 1181783 A	13/05/98
		EP 0871721 A	21/10/98
		NO 974123 A	07/11/97